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THE UNIVERSITY OF ALBERTA

ARACHIDONIC ACID METABOLISM IN CEREBRAL ARTERIES: RELEVANCE TO CEREBRAL VASOSPASM

BY

RICHARD SCHULZ

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHARMACOLOGY

EDMONTON, ALBERTA

FALL, 1989

MARCH 30, 1989

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Dear Rick,

I hereby authorize you to include material from the paper(s) entitled:

"Effects of vasospasm on levels of prostacyclin and thromboxane A_2 in cerebral arteries of the monkey."

published in:

Neurosurgery 22:45-50,1988

of which I am co-author, into your Ph.D. dissertation.

Sincerely yours,

Bryce Weir

MARCH 30, 1989

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Sincerely yours,

M. Grace

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Dear Rick,

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3. "Effects of vasospasm on levels of prostacyclin and thromboxane A_2 in cerebral arteries of the monkey".
4. "Lipoxygenase product formation by cerebral arteries".

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1. Proceedings of the Western Pharmacolomy Society 29: 101-104 (1986)
2. European Journal of Pharmacology 136: 345-352 (1987)
3. Neurosurgery 22: 45-50 (1988)
4. Submitted for publication

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Sincerely yours,

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Professor and Chairman

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3. in Cerebral Vasospasm (R. Wilkins, ed.), New York: Raven Press, pp. 259-264 (1988).
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External Examiner

25 April 1989

To my grandfather -

Daniel Koch

for his jovial inspiration

ABSTRACT

Following the rupture of a cerebral aneurysm a blood clot forms in the subarachnoid space. About 3-7 days later a narrowing of the arteries in the region of the clot may occur, which can result in ischemic damage to the portion of the brain supplied by the affected blood vessel. This often fatal condition is resistant to direct pharmacological intervention.

The metabolites of arachidonic acid have been implicated in the pathogenesis of this disorder. They consist of the cyclooxygenase enzyme products, which include the prostaglandins, prostacyclin and thromboxane A_2, and those formed by the lipoxygenase enzymes, including the leukotrienes and the hydroperoxy- and hydroxy-eicosatetraenoic acids (HPETEs and HETEs).

Using an animal model of vasospasm, freshly isolated cerebral arteries, and smooth muscle and endothelial cells cultured from this source, the synthesis of arachidonic acid metabolites by the cerebral arterial wall was studied. The ability of cerebral arteries to synthesize both the vasodilator prostacyclin and its physiological antagonist thromboxane was investigated in a primate model of vasospasm. After exposure for seven days in vivo to a subarachnoid blood clot, such arteries showed an altered ability to synthesize prostacyclin as compared to vessels obtained from animals in which blood clot had been removed 24 hours after its placement. No differences in thromboxane production were observed.

Freshly isolated canine cerebral arteries showed a sustained contraction to arachidonic acid which was not affected by aspirin-like

drugs, but was attenuated by inhibitors of lipoxygenase formation and action. Analysis of normal cerebral arteries incubated in vitro with arachidonic acid or with the calcium ionophore A23187 revealed that they can synthesize both HPETEs and leukotrienes.

To investigate lipoxygenase metabolite formation at a cellular level, both smooth muscle and endothelial cells were cultured from cerebral arteries. Smooth muscle cells, when stimulated with arachidonic acid, produce 15-HETE as their primary lipoxygenase metabolite.

Thus, the cerebral blood vessel wall possesses rich lipoxygenase activity. The range of biological activities of the HPETEs and the leukotrienes can account for many features of cerebral vasospasm. Pharmacological manipulation of their release and action may be beneficial in the treatment of this disorder.

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ABBREVIATIONS

AA	arachidonic acid (5,8,11,14-eicosatetraenoic acid
Abs	absorbance
ASA	acetylsalicyclic acid
CSF	cerebrospinal fluid
ED_{50}	median effective dose
EDRF	endothelium-dependent relaxant factor
EDTA	ethlyenediaminetetraacetic acid
ETYA	5,8,11,14-eicosatetraynoic acid
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HETE	hydroxyeicosatetraenoic acid
HHB	Hanks'-HEPES buffer
HPETE	hydroperoxyeicosatetraenoic acid
HPLC	high performance liquid chromatography
5-HT	5-hydroxytryptamine (serotonin)
KBB	Krebs-bicarbonate buffer
LT	leukotriene
LXA	lipoxin A
LXB	lipoxin B
metHb	methemoglobin
NDGA	nordihydroguaiaretic acid
oxyHb	oxyhemoglobin
PBS	phosphate-buffered saline
PG	prostaglandin
PGI_2	prostacyclin
S.E.	standard error of mean of sample

SAH	subarachnoid hemorrhage
SRS-A	slow reacting substance of anaphylaxis
TX	thromboxane
UEA-I	*Ulex europaeus* agglutinin-I
UV	ultraviolet

CHAPTER 1

INTRODUCTION

CHAPTER 1

INTRODUCTION

Following the rupture of an aneurysm in the cerebral circulation a delayed onset and long lasting contraction of the arteries surrounded by perivascular blood clot can occur. This disorder, known as cerebral vasospasm, provides the focus and the primary motivation behind the work to be presented in this thesis. The introduction is divided into three main sections: I. a brief explanation of the condition, some of the experimental models used to study this disorder, and some putative mechanisms of its pathogenesis, II. a discussion of the role of the eicosanoids in cerebral vasospasm, and III. a guide to the questions which were examined during the work presented in this thesis. In this chapter and indeed, throughout the experimental studies performed which comprise the body of this thesis, I would like to present the hypothesis that the metabolites of arachidonic acid, known collectively as the eicosanoids, may play a significant role in the development of this debilitating condition.

I. The Disorder Known as Cerebral Vasospasm

A. Definition

The precipitating factor in the development of the disorder known as cerebral vasospasm or alternately cerebrovascular spasm is the rupture of a blood vessel in the cerebral circulation, usually at a site where the arterial wall has been weakened. This is most often at the site of an aneurysm, a sac-like bulging of the blood vessel where the integrity of the internal elastica lamina and medial layers of the blood vessel wall has been compromised (Weir, 1987 p. 224). Aneurysms are

most often found at the bifurcations of the cerebral arterial tree, where shear stress from turbulent blood flow is at its greatest. The cause of rupture is most commonly associated with episodes of elevated blood pressure, and affects some 28,000 individuals in North America each year, with over 19,000 dying or being disabled as a result (Kassell et al., 1985). Rupture commonly occurs at the internal carotid, anterior or middle cerebral arteries or in the communicating arteries of the circle of Willis (Weir, 1987 p. 344).

The circle of Willis is the unique polygonal network of arteries at the base of the brain where blood supplied by the pair of internal carotid arteries and the basilar artery, which arises from the junction of the vertebral arteries, join to ensure uninterrupted blood flow to the brain (see Grey [1901] 1978, p. 507 for a picture). This insures that if flow from one of the three major supply networks to the brain is disturbed, the circulation to the brain is maintained. However it is ironic that the convuluted anastomoses and bifurcations which the circle of Willis presents make it the most frequent site of aneurysm formation.

With the rupture of the so-called berry aneurysm, blood escapes into the intracranial space underneath the subarachnoid membrane which envelops the central nervous system. The extravasated blood then clots and comes into intimate contact with the adventitial surface of cerebral arteries. This is commonly referred to as subarachnoid hemorrhage (SAH). The presence of blood in the subarachnoid space is acknowledged as the precipitating factor in the development of cerebral vasospasm. Within a number of days following the rupture, a

constriction develops close to the site of the aneurysmal rupture (Niizuma et al., 1979), but can also extend to other regions of the cerebral vasculature. This delayed onset arterial narrowing is a hallmark feature of this condition, and thus is often described as chronic cerebral vasospasm. This must be distinguished from "acute" or "early" vasospasm, a narrowing of the cerebral arteries observed within the first few hours after blood application seen in experimental models of vasospasm (vide infra). Whether the acute vasospasm is present in clinical vasospasm is less well established (Weir et al., 1978; Odom, 1974; Wilkins, 1976).

Weir et al. (1978) described the time course of chronic vasospasm in man as having its onset on day 3 following SAH, reaching a maximum in intensity about day 7, and, if resolution occurs, this happens by the second week after the rupture.

There are two definitions of cerebral vasospasm, one which is based on the clinical presentation of the disorder and the second, based on angiographic evidence of arterial narrowing (Kassell et al., 1985). Clinical vasospasm is the consequence of ischemia to the region of the brain affected by the arterial narrowing, and manifests itself in impairments of consciousness, speech and motor activity and, if severe enough, results in death or permanent neurological deficits. Angiographic vasospasm is the narrowing of some portion of arterial tree, visualized by the injection of radio-opaque dye into the cerebral circulation and X-ray photography. In general the time course of clinical and angiographic vasospasm overlap, although 70% of those individuals with cerebral vasospasm may show arterial narrowing, only 20-30% will

have neurological deficits as a result (Kassell et al., 1985). The degree and location of the arterial narrowing, as well as the capacity of collateral circulation to compensate best explains this phenomenon.

It is widely agreed that cerebral arteries, once in vasospasm, are refractory to any pharmacological treatment. Wilkins (1980, 1986) provided an exhaustive review of the literature covering all attempted drug treatments. Vasodilators (with direct or indirect mechanism of action), anti-fibrinolytic agents, drugs or procedures which neutralize the vasospastic effects of clotted blood, drugs which reduce focal acidosis, and procedures or agents which interfere with sympathetic monoaminergic pathways have all been unsuccessful in treating cerebral vasospasm.

Weir (1987) has published an exhaustive treatise covering all aspects related to aneurysms in the nervous system. The reader is also referred to excellent review articles covering the subject of cerebral vasospasm from a clinical standpoint (Kassell et al., 1985; Weir, 1987, pp.505-569) as well as from a pharmacologist's perspective (Cook, 1984; Bevan and Bevan, 1988).

B. Experimental Models in the Study of Cerebral Vasospasm

Investigation of the complex nature this disorder has been complicated and perhaps confounded by the lack of an entirely suitable animal model which accurately reflects the situation in humans. The most apparent difficulty is in reproducing the late onset vasospasm where delayed neurological deficits occur and can be observed. There are a variety of in vitro approaches which are becoming more informative and these include (in order of their prevalence to date),

pharmacological studies of blood vessel contractility using vessel segments or perfused arterial preparations, biochemical investigations of the artery and most recently studies using cells isolated from cerebral arteries.

The shortcoming of all the in vitro studies is that none of the methodologies specifically address the delayed cerebrovascular spasm and thus may only provide us with knowledge of acute vasospasm which is of questionable clinical significance. However Bevan and Bevan (1988) have put forward the hypothesis that the delayed constriction of cerebral arteries in spasm is not an active contraction but a passive, irreversible state as a result of processes which begin during the early stages following subarachnoid hemorrhage. Thus the severity of initiating events, which may include excessive active contraction of the artery exposed to a number of vasoactive agents released during clot formation, could initiate damage to cells in the arterial wall and thus begin a decline in vessel function culminating in irreversible passive vasoconstriction. Although evidence for this acute vasospasm in humans is absent or minimal, this hypothesis places in vitro studies in a more useful light.

Animal models. The animal model which most closely approximates the human condition is that using the cynomolgus monkey, in which either an autologous blood clot is surgically packed around the middle cerebral artery, a method which was developed at this university (Espinosa et al., 1984; Nosko et al., 1985), or where a needle, previously passed through the wall of the internal carotid artery, is withdrawn, causing hemorrhage in the subarachnoid space (Frazee,

1982). As summarized by Weir (1987, p.568) from a series of 54 monkeys, none showed any neurological deficits immediately after clot placement. Within a few days, 80% of the animals showed signs of severe angiographic vasospasm. Three animals in all showed signs of delayed ischemic deficits, two on day 4 and one on day 5 post-SAH. This is reported to be the first unequivocal example of delayed ischemic deficits associated with vasospasm occurring in an animal model of vasospasm (Weir, 1987 p. 568). This is the same model which was used in the experiments discussed in Chapter 2 of this thesis.

A more prevalent although less suitable model involves direct injection of blood into the subarachnoid space of dogs, a process which, in some models, is repeated after a few hours (Wilkins and Levitt, 1970; Liszczak et al., 1983). In the model of Liszczak et al. (1983), blood was injected twice, at a time interval of 48 hours. Development of vasospasm is then more reliable and mimics certain aspects of the human condition. For example vessels in spasm do not respond to drug treatment, ultrastructural changes such as endothelial damage and smooth muscle cell alterations were observed, and both angiographic and morphological vasospasm appeared where blood elements were found in arterial adventia (Liszczak et al., 1983). A common observation in the canine model is the development of acute vasospasm within a few hours after blood injection.

Studies of blood vessel contractility. Studies measuring the development of tone in isolated blood vessel segments are a useful companion for observations made in animal studies, if one realizes their limitations. It provides a methodologically convenient approach, in

contrast to the surgical process, which involves expense and effort in obtaining serial angiograms of an animal which must be observed over a number of days. However, measurement of the contractile response to a putative spasmogen may only reflect conditions soon after SAH, although this acute "vasospasm" may be an important investigative approach according to the theory of Bevan and Bevan (1988) (vide supra). It is possible that a substance which may be released by the clot itself and/or the cerebral artery wall, could cause a slowly developing contraction. However this putative mediator must have either a very slow and prolonged release or have an extremely long duration of action (days or weeks). As stated by Cook (1984):

> "Unfortunately, our present knowledge of pharmacology does not encourage a belief in this sort of compound. Irreversible antagonists are relatively common, but irreversible agonists are essentially unknown. Equally, it seems that nearly all receptor systems show some measure of tachyphylaxis, desensitization or autoregulation which interferes with what otherwise would be an indefinitely sustained contraction."

The likelihood that cerebral vasospasm is the result of a single pharmacological agent, either via the presence of a contractile agent or loss of an endogenous vasodilator, is remote.

What is important is the testing of putative spasmogens on isolated cerebral artery segments to determine their biological activity and see if they cause the release of any further spasmogens from the cerebral artery wall itself. For example, little is known of the capacity of the cerebral vasculature to release eicosanoids, especially those products of the lipoxygenase enzymes.

Contractility studies allow a very efficient assessment of the relative contributions of both endothelium and smooth muscle to the pharmacological response of the intact artery. Furchgott and Zawadzki (1980) described an endogenous vasodilator released from the endothelium as the cause of the dilatory response of blood vessels to acetylcholine. This simple observation provoked a revolutionary change in the conceptual approach of blood vessel pharmacologists. The blood vessel not only possessed receptors in smooth muscle but a very important luminal sheet of cells which modulated the blood vessel response to a variety of agents. This also explains the common divergence of results obtained between groups working with the same blood vessel preparation from the same species. It is only a matter of experimental technique in preparing the arteries for study of contractility which may result in a large denudation of the endothelium (Furchgott, 1983). More important is a common observation that various degrees of endothelial damage, from vacuolization to denudation, are evident in arteries which have been in spasm (Fein et al., 1974; Hughes et al., 1978; Tanabe et al., 1978; Liszczak et al., 1983). The function of the endothelial cell in the development of cerebral vasospasm is now under intense investigation, and studies of isolated cerebral arteries have helped to delineate its role.

Information which is lost during investigation of the pharmacological responses of rings or strips of cerebral artery is the effect of extraluminal versus intraluminal application of spasmogens, which is an issue of great significance to the study of cerebral vasospasm. Not only is the presence of blood clot on the adventitial surface a prime

indication of spasmogenic outcome clinically, it may also explain aspects of the time course of development of vasospasm in terms of diffusion of mediators through the arterial wall. As well, the role of the endothelium in modulation of the response is dependent upon the site of application of putative spasmogens (Toda et al., 1988). Thus a number of new studies have now looked at the in vitro responses of cerebral arteries using perfusion methods and application of contractile agents such as blood or hemoglobin to the adventital side (Tsuji and Cook, 1989; Vinall and Simeone, 1988; Lang and Maron, 1988).

Biochemical studies. Investigation of the possible mechanisms of cerebrovascular spasm at a biochemical level is lacking in comparison with the number of studies performed using peripheral arteries in investigations of diseases such as atherosclerosis and hypertension. This however is changing as studies of receptor binding (Tsukahara et al., 1986), subcellular calcium compartmentalization (Barry et al., 1985), and investigations of arachidonic acid metabolism appear (vide infra). To date no investigation of signal transduction specifically relating to phosphoinositide metabolism and/or protein kinase regulation at the subcellular level has been undertaken using normal or spastic cerebral arteries.

Studies using cells from cerebral arteries. This area is completely new in the study of cerebral vasospasm, as until recently no culture methods existed for the isolation of cells from cerebral arteries. A recent study by Fujii and Fujitsu (1988) emphasized the need for cultures of cerebral artery-derived cells, as they showed that smooth muscle cells isolated from the aorta underwent a progressive

contraction and ultrastructural changes resembling myonecrosis upon long-term incubation with hemoglobin. The area is now being investigated along two fronts, one approach being the use of freshly isolated smooth muscle cells from cerebral arteries to characterize their electrophysiological properties in response to spasmogens (Steele, Stockbridge and Weir, in preparation). The other is the isolation and serial cultivation of cerebral artery smooth muscle cells and endothelial cells for studies of arachidonic acid metabolism, as well as for their ion current activities. Chapter 3 is concerned with the isolation, culture and identification of cerebral artery smooth muscle and endothelial cells. There is only one other reference to the culture of endothelial cells from the main conduit arteries of the cerebral circulation (Goetz et al., 1985) and no references to the culture of smooth muscle cells from this source.

In contrast, interest in the properties of the blood-brain barrier prompted the culture of cells from the cerebral microvasculature, including endothelial (DeBault et al., 1981; Spatz et al., 1980) and smooth muscle cells (Diglio et al., 1986). Microvascular endothelial cells have already been subjected to studies of their arachidonic acid metabolism (Moore et al., 1988 a,b). The nature of the lipoxygenase product profile of arachidonic acid metabolism in cerebral artery smooth muscle cells is the subject of Chapter 7.

With the ability to culture cells from cerebral arteries we can now apply various biochemical and electrophysiological means to the study of vasospasm. Contractility studies which implicate endothelium-mediated processes can be complemented with the study of endothelial

cells from cerebral artery for the release of mediators such as prostacyclin (Goetz et al., 1985) and other metabolites of arachidonic acid. Endothelium-derived relaxation factor (EDRF) or the newly discovered polypeptide endothelin, made by endothelial cells and reputed to be one of the most potent spasmogens of peripheral arteries (Yanagisawa et al., 1988) can now be investigated directly . Signal transduction studies relevant to the cerebral circulation will now be attainable. Not to be underestimated is the possibility to perform experiments with smooth muscle and endothelial cells in controlled co-culture systems, to better understand how each cell modifies the response of the other in the production of the integrated vascular response (van Buul-Wortelboer et al., 1986; Ganz et al., 1986).

C. **Agents implicated to cause cerebral vasospasm**

As Cook (1984) has written, there are only three logical sites to look for a spasmogen: the blood (fresh and/or clotted), the cerebrospinal fluid, and the arterial wall. The agent or agents must be present as some part of the cerebral circulation, normally found in an inactive form, and some plausible mechanism must explain their release and/or activation. Vasospasm after hemorrhage in cerebral arteries is a unique phenomenon; in no extracerebral site does hemorrhage cause such a constriction. This places a great emphasis on the qualities of the space filled by the cerebrospinal fluid, and mechanisms which remove the blood clot from the subarachnoid space. CSF has poor fibrinolytic capacity (Weir, 1987 p. 91) and the rate of flux of the CSF through the cisterns is low. Simplistically, the agent could have a spasmogenic effect or interfere with the production of a

vasodilator which is normally present. The most important agents which are or have been implicated to be involved in the pathogenesis of cerebral vasospasm include catecholamines, serotonin, blood products such as hemoglobin, activated oxygen species, and metabolites of arachidonic acid. All of these will be discussed briefly in this section of the thesis except for the eicosanoids, which shall be discussed in more detail in Section II of this chapter.

Catecholamines. Peripheral arteries contract in response to stimulation of α-adrenoceptors and dilate via activation of β-adrenoceptors. Cerebral arteries have been shown to possess adrenergic innervation (Nielsen and Owman, 1967; Lee et al., 1980) although the arteries show only a weak constriction to agonists which act at α-adrenoceptors. The "denervation supersensitivity" hypothesis arose from the combination of results where a marked reduction in catecholamine-related fluorescence was seen in cerebral arteries exposed to subarachnoid clot (Peerless and Kendall, 1975; Duckles et al., 1977; Lobato et al., 1980), whereas catecholamine levels are known to rise during vasospasm (Loach and Benedict, 1980). However, treatment of vasospasm with α-antagonists such as phenoxybenzamine (White, 1980) and phentolamine (Walter et al., 1980) was unsuccessful and this line of reasoning has largely been abandoned.

5-Hydroxytryptamine. This potent constrictor of cerebral arteries, also known as serotonin or 5-HT, is stored in platelets and is released upon platelet activation. It was the subject of intense investigation, since one of the unique features of the cerebral circulation is that cerebral arteries are much more sensitive to 5-HT than

peripheral arteries (Owman et al., 1978). Using a model of vasospasm in the monkey, Boisvert et al. (1979) reported that a transient constriction was seen when 5-HT was injected into the subarachnoid space with blood, but that the delayed vasospasm was insensitive to the 5-HT receptor antagonist, cyproheptadine. The contraction of cerebral arteries in vitro in response to blood is not inhibited by 5-HT antagonists (Boullin et al., 1978; Okwuasaba et al., 1981b), and arteries exposed to 5-HT show a rapid desensitization (Okwuasaba et al., 1979). It is currently believed that 5-HT contributes only to the acute phase of vasospasm, if at all.

Blood-derived products. As already mentioned, there is a close association between the presence of blood in the subarachnoid space and the development of cerebral vasospasm as shown using animal models. The volume of blood clot, which can be measured using computed tomography scanning (Fisher et al., 1980) is also related to the severity of vasospasm. It seems very probable that there must be some component(s) of blood which is (are) either released or synthesized after hemorrhage and is (are) responsible for the production of vasospasm. The evidence supporting this idea is as follows:

1. Bloody CSF from patients with vasospasm is known to contract cerebral arteries (Boullin et al., 1976; Okwuasaba et al., 1981b) although the latter study showed that CSF from patients with hemorrhage but not showing signs of vasospasm has nearly the same activity. Samples of normal CSF did not have any constrictor effect.

2. Cerebral arteries are much more sensitive than peripheral arteries to the constrictor effects of blood products such as hemoglobin (Toda et al., 1980) and this is not due to any seritonergic mechanism.
3. Intact erythrocytes do not contract cerebral arteries in vitro whereas the lysed cells have intense activity (Osaka, 1977). When fresh blood is stored, its vasospastic activity follows a time course which approximately parallels red cell lysis and the onset of vasospasm (Sonobe and Suzuki, 1978; Okuwasaba et al., 1981a). Biochemical analysis of hemolyzed red blood cells by molecular exclusion chromatography (Cheung et al., 1980) showed that the vasoactive fraction had a molecular weight of 40 to 45 kD and that the constrictor activity resided in a protein.

Most of the research efforts have focused on the effects of hemoglobin itself (for detailed review see Weir, 1987, pp.518-523). Hemoglobin (oxyhemoglobin or oxyHb) is a hemeprotein having the highest capacity to bind oxygen with its central iron atom in the ferrous state (Fe^{2+}). If this molecule is oxidized, which occurs spontaneously both in vitro and in vivo when it is released from a lysed red blood cell, the iron atom loses an electron and goes to the ferric state (Fe^{3+}). This molecule, which has a greatly reduced capacity to bind oxygen, is now called methemoglobin (metHb). The potential mechanisms by which hemoglobin may be involved in cerebral vasospasm are as follows: 1. by the inhibition of endothelium-derived relaxation factor (EDRF), 2. by the formation of activated oxygen

species during its degradation from oxyHb to metHb and 3. by the stimulation of products of the arachidonic acid cascade. The first two points will be discussed below, the last will be reserved for Section II of the Introduction.

The endothelium is known to release a labile substance in response to stimulation with a wide variety of agonists, as well as to alterations in blood flow. This compound acts upon the underlying smooth muscle to cause vasodilatation (Furchgott and Zawadzki 1980; Förstermann and Neufang, 1984; Rubanyi et al., 1986; Griffith et al., 1987). The agent, first described by Furchgott and Zawadzki (1980), was given the name, "endothelium-dependent relaxant factor" (EDRF). It acts by stimulating guanylate cyclase to increase cyclic GMP content of smooth muscle cells (Holzmann, 1982; Rapoport and Murad, 1983) and is inhibited by hemoglobin and methylene blue (Martin et al., 1985a). Considering both the endothelial damage evident in vessels exposed to blood clot, and the specific inhibition by hemoglobin, the question arose whether lack of this vasodilator or inhibition of its effect could explain the development of vasospasm (Bowman, 1985).

Nakagomi et al. (1987 a,b) studied the ability of rabbit basilar arteries, exposed to subarachnoid clot, to relax to acetylcholine. They showed that there was some impairment of the ability of these arteries to release EDRF. Using CSF samples obtained from patients five days after subarachnoid hemorrhage, Kanamaru et al. (1988) showed that the relaxant response of canine basilar arteries to the calcium ionophore A23187 was inhibited. It is generally believed,

however, that the inhibition of EDRF release plays only a minor role in the development of vasospasm.

A hypothesis which requires further investigation is that concerning the generation of free radicals in the subarachnoid space following SAH (Sasaki et al., 1979; Sano, 1988). One source of free radicals could be in the autooxidation of hemoglobin to methemoglobin. This could be the initiating reaction in the propogation of activated oxygen species, such as hydroxyl radical, superoxide anion, and lipid peroxyl radicals. The type and variety of non-specific damage such species could bring, as well as a prolonged time course which would be expected to correlate with clot breakdown, makes this theory an attractive one. The normal serum defence mechanisms against these harmful species, such as glutathione peroxidase, catalase and the antioxidant, vitamin E are low in human CSF (Cohen, 1983). In a study of 25 patients with ruptured intracranial aneurysms, it was shown that the concentration of lipid peroxides in the CSF of patients suffering from vasospasm was higher than control samples and that the levels rose in the first 12 days after bleeding. The activity of superoxide dismutase in CSF was diminished 5 days post-SAH, whereas catalase levels appeared to rise during the first week (Sakaki et al., 1986).

The involvement of free radical reactions in the pathogenesis of vasospasm is even more compelling when considered in combination with the participation of arachidonic acid metabolites. This idea will be presented more fully in the following section of the Introduction.

II. Role of Eicosanoids in the Development of Cerebral Vasospasm

A. The arachidonic acid cascade

Historical aspects. The 20-carbon fatty acid arachidonic acid (AA, or 5,8,11,14-eicosatetraenoic acid) is the most common fatty acid found in the phospholipid of mammalian cells. All products of its metabolism are collectively known as the eicosanoids. These include the prostaglandins, thromboxane, leukotrienes, lipoxins, hydroperoxy- and hydroxy- eicosatetraenoic acids (HPETEs and HETEs). It is now realized that all cells have the capacity to synthesize these biologically relevant products. No other class of endogenous compounds have generated such universal interest in biological circles. All the prostaglandins have in common the fact that they are synthesized by a complex of microsomal enzymes referred to as fatty acid cyclooxygenase.

Nearly sixty years ago Kurzrok and Lieb (1930) first noted the contractile and relaxant effects of human semen on isolated uterine strips. It was von Euler (1935) who proposed the term "prostaglandin" to describe the lipid soluble-acid which comprised the active principle. It was not until some thirty years later with the crystallization of two prostaglandins of the E and F series that it was demonstrated that the prostaglandins were indeed a family of compounds with unique chemical structures. (see Bergström and Samuelsson, 1968). Until recently it was believed that prostaglandin E_2 (PGE_2) and $PGF_{2\alpha}$ were the major prostaglandins. Research in the 1970's, however, led to the discovery of the unstable cyclic endoperoxides, PGG_2 and PGH_2 which, in turn, led to the discovery of new prostaglandins with potent

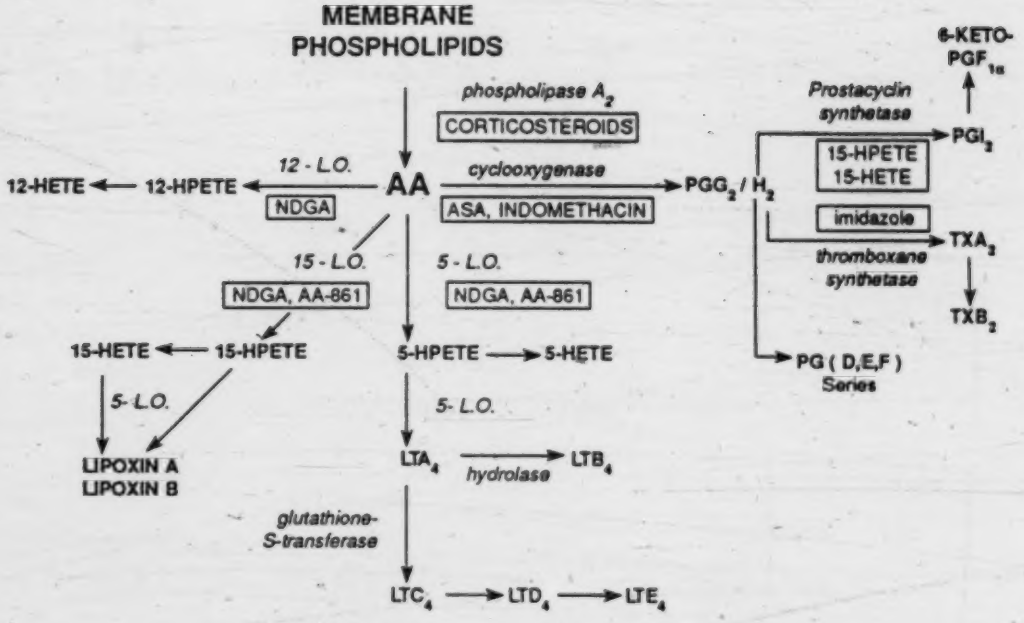

Figure 1. Major routes of arachidonic acid metabolism. Inhibitors are shown in boxes.
***L.O.* = lipoxygenase**

vascular activities, thromboxane A_2 (TXA_2) (Hamberg et al., 1975), and prostacyclin (PGI_2) (Moncada et al., 1976).

In 1938, Feldberg and Kellaway showed that a substance causing the slowly developing and tonic contraction of guinea-pig intestine was released from lung tissue stimulated by cobra venom, which could also be formed during anaphylactic reaction of the lung (Kellaway and Trethewie, 1940). Brocklehurst coined the term "slow-reacting substance of anaphylaxis" and the chemical identification of this principle as a mixture of leukotrienes was not unravelled until the late 1970's (see Samuelsson, 1983).

Pathways of eicosanoid formation. The products of the arachidonic acid cascade (Fig. 1) are not stored in cells, they are synthesized de novo in response to a diverse range of physical, hormonal and chemical stimuli. Arachidonic acid is esterified to the phospholipids which make up the cell membrane. Upon stimulation there is a rise in intracellular calcium which results in the activation of acyl hydrolases, primarily phospholipase A_2, which acts to increase the level of free arachidonic acid in the cell. Depending upon the cell type in which this occurs, the free acid is then rapidly metabolized to a variety of products by distinct reactions, the main pathways of which are distinguished by the two families of enzyme systems involved, either the cyclooxygenase or lipoxygenase. Products containing ring structures, the "primary" prostaglandins (A to F series), the cyclic endoperoxides (G,H), prostacyclin (I) and thromboxane (TX), result from the initial action of cyclooxygenase. Straight-chain oxygenated derivatives of arachidonate are formed by the action of

lipoxygenases, which are differentiated into 5-, 12-, and 15- series by the initial site of attack of oxygen on the fatty acid chain. This leads to the intermediate hydroperoxyeicosatetraenoic acids (HPETEs) which show important biological actions. These unstable compounds can be reduced spontaneously in an aqueous environment or by the action of peroxidase to the corresponding hydroxyeicosatetraenoic acid (HETE). Alternatively 5-HPETE is the substrate for further enzymatic reactions which lead to the leukotrienes and lipoxins (Samuelsson, 1983; Samuelsson et al., 1987).

Eicosanoid inhibitors and antagonists. Inhibition of the formation of products or effects of the arachidonic acid metabolites can be achieved in numerous ways. By inhibiting the enzyme phospholipase A_2 the release of arachidonic acid and hence all metabolites thereof is blocked. The corticosteroids have this property, and their mechanism of action is indirect by stimulating the synthesis of an endogenous anti-inflammatory protein termed lipocortin (Wallner et al., 1986). How lipocortin actually inhibits phospholipase activity is in dispute since it is identical in structure to a calcium and phospholipid binding protein named calpactin. It has been suggested that inhibition is achieved by the binding of the protein to the substrate and not by a direct action on the enzyme itself (Davidson et al., 1987). The inhibitory action of recombinant lipocortin on eicosanoid release has been verified, as it blocks the release of thromboxane A_2 into perfused guinea pig lungs stimulated with leukotriene (Cirino et al., 1987).

A milestone was reached when Vane (1971) discovered that the aspirin-like drugs acted by inhibiting prostaglandin formation, via

irreversible inhibition of fatty acid cyclooxygenase. As this results in the blockade of formation of the cyclic endoperoxides, all subsequent prostaglandins are reduced in quantity. Compounds such as imidazole, dazoxiben, OKY-046, and OKY-1581 were developed as selective inhibitors of thromboxane synthetase, with the idea that the production of beneficial prostaglandins such as prostacyclin would not be affected (see Smith, 1987). Alternatively, antagonists of thromboxane receptors such as BM 13.177 have also been synthesized (see Moncada and Higgs, 1986 for references).

Although potentially useful in treating inflammatory disorders such as asthma and psoriasis, no lipoxygenase inhibitor has yet been applied clinically to date. There are, however, a variety of lipoxygenase inhibitors which are useful tools to investigate this arm of arachidonate metabolism. Compounds such as nordihydroguaiaretic acid (NDGA) (Tappel et al., 1953), AA-861 (Yoshimoto et al., 1982), and U-60,257 (Bach et al., 1982) are inhibitors of lipoxygenase. Agents like BW 755c (Blackwell and Flower, 1978) and 5,8,11,14-eicosatetraynoic acid (ETYA) (Tobias and Hamilton, 1978) can inhibit both lipoxygenase and cyclooxygenase pathways. Antagonists of leukotriene receptors include FPL 55712 (Augstein et al., 1973), first described as an antagonist of slow reacting substance of anaphylaxis and now recognized as a specific antagonist of LTD_4 receptors (Vane and Botting, 1987), and a large number of agents being sought after by pharmaceutical houses, for example L-649,923 (Jones et al., 1986).

The following discussion of the cellular sources of the eicosanoids, their pharmacological actions and inhibition of their synthesis

will focus on the blood vessel itself. For further information on their biosynthesis and pharmacological actions, several excellent reviews are available (see Samuelsson, 1983; Samuelsson et al., 1987; Moncada et al., 1985; Moncada and Higgs, 1986; Leslie et al., 1985).

Actions and formation of cyclooxygenase products in the vasculature. From the action of prostacyclin synthetase on the cyclic endoperoxide PGH_2, the blood vessel wall produces prostacyclin as its major cyclooxygenase product. This compound has a half-life of only 2-3 minutes and is degraded to 6-keto $PGF_{1\alpha}$ which can be directly assayed for. The intimal lining is the richest source of this vasodilator and its synthesis decreases progressively as one moves to the adventitia (Moncada et al., 1977). Cultured endothelial cells produce prostacyclin as their major cyclooxygenase derived product (Weksler et al., 1977). This product has potent anti-aggregatory effects on platelets and is a dilator of blood vessels, including the cerebral arteries (Boullin et al., 1979; Chapleau and White, 1979; Toda, 1980). Its physiological antagonist is TXA_2, a prostanoid with platelet aggregatory and vasoconstrictor activity which is synthesized primarily by the platelet (Hamberg et al., 1975). With a half-life of only 30 seconds it is rapidly converted to the inactive metabolite, TXB_2. Vascular tissue can also synthesize TXA_2 (Neri Seneri et al. 1983). Cultured endothelial cells but not smooth muscle cells from bovine aorta can make TXA_2, although PGI_2 is still produced in a five to tenfold greater amount (Ingerman-Wojenski et al., 1981). The role of thromboxane production from vascular tissue is unclear. Other prostaglandins such as $PGF_{2\alpha}$, PGE_2, and PGD_2 have been shown to be

released from vascular tissue albeit as minor products (Neri Seneri et al., 1983).

Actions and formation of lipoxygenase products in the vasculature. The lipoxygenase pathway was long known in plants but it was not until recently that it was discovered in mammals. The most well characterized lipoxygenase is the 5-lipoxygenase, which catalyzes oxygenation at the C-5 position of the arachidonic acid chain. The first intermediate is 5-hydroperoxyeicosatetraenoic acid (5-HPETE), which is further catalyzed by the same enzyme to form the unstable epoxide, leukotriene A_4. This second intermediate, akin to the cyclic endoperoxide PGH_2 of the cyclooxygenase pathway, can be transformed to a variety of products, depending upon the cell type and the particular enzymes activated. Blood cells such as leukocytes contain a hydrolase which acts on the the epoxide ring to form 5,12-dihydroxyeicosatetraenoic acid (LTB_4), an important chemotactic factor for these cells and stimulates aggregation, enzyme release, and generation of superoxide anion from neutrophils (see Ford-Hutchison and Letts, 1986). Lung tissue, mast cells, and macrophages are well known to generate LTC_4 by addition of glutathione to LTA_4 by action of a glutathione-S-transferase. This is the first in a series of three "peptidoleukotrienes", including LTD_4 and LTE_4, which are known for their myotropic effects. A glutamic acid residue is lost in the conversion of LTC_4 to LTD_4 and a further cleavage of glycine results in LTE_4. The slow-reacting substance of anaphylaxis is now recognized to be a mixture of LTC_4 and LTD_4. Other lipoxygenases such as 12- and 15-lipoxygenase convert arachidonic acid to 12-HPETE and

15-HPETE, respectively. These lipid hydroperoxides have potent biological activity in the vascular system (vide infra). The newest members of the lipoxygenase pathway are the lipoxins, which are formed by the actions of both 5- and 15- lipoxygenases and are essentially a variety of trihydroxyeicosatetraenoic acid derivatives (see Samuelsson et al., 1987 for review).

A 5-lipoxygenase system is present in vascular tissues of peripheral arteries (Piper et al., 1983; Piper and Galton, 1984; Piper et al., 1988; Wittman et al., 1987). In response to incubation with the calcium ionophore A23187, Piper and Galton (1984) have shown that porcine pulmonary arteries release peptidoleukotrienes and LTB_4. LTC_4 and LTD_4 are quickly metabolized to LTE_4 in this system. In contrast to the vascular wall gradient of prostacyclin releasing activity, the adventitia releases the highest amount of peptidoleukotrienes, although the intima and media can also release these substances (Piper and Galton, 1984). Comparing various porcine arteries the cerebral arteries have the greatest capacity to synthesize LTs, five to nine-fold higher than renal, pulmonary and coronary arteries (Piper et al., 1988). The order of potency of the myotropic action of the peptidoleukotrienes is $LTC_4 > LTD_4 > LTE_4$. Depending upon the vascular bed these substances can cause constriction, as in coronary (Burke et al., 1982) and cerebral arteries (Tagari et al., 1983; Jancar et al., 1987- Chapter 4) or dilatation in skin blood vessels (Bisgaard et al., 1982).

The HPETEs and HETEs have important vascular actions of their own. The first tissue where lipoxygenase activity was discovered was

in platelets, where 12-HETE, the stable breakdown product of its precursor 12-HPETE, is the major product (Hamberg and Samuelsson, 1974). It is known for its chemotactic activity in attracting polymorphonuclear leukocytes. Greenwald et al. (1979) discovered that vascular tissue had the ability to synthesize HETE, the position of the hydroxyl group not determined at the time. Human umbilical arteries were shown to have the ability to synthesize both 15- and 11-HETE from arachidonic acid, the former being the major lipoxygenase metabolite in the tissue and the latter being a cyclooxygenase product (Setty and Stuart, 1986).

The ability of the blood vessel wall to release HPETEs and HETEs is of interest since it is known that 15-HPETE has the ability to inhibit prostacyclin formation from blood vessels (Gryglewski et al., 1976), thus representing a possible endogenous regulator of prostacyclin release. This same compound however does not affect thromboxane synthetase (Ham et al., 1979) and could be a possible clue to the view of an imbalance in prostacyclin versus thromboxane production hypothesized in a number of pathophysiological states.

Fatty acid peroxides prepared from the incubation of polyunsaturated fatty acids, including arachidonic acid, with soybean lipoxygenase, have the ability to contract rabbit aortic strips with a greater efficacy and potency than their precursors (Asano and Hidaka, 1979). If arachidonic acid is used as the substrate, this reaction produces 15-HPETE. These contractions were not reduced with inhibitors of cyclooxygenase suggesting that the mechanism of action of the HPETEs is not through the stimulation of prostaglandin synthesis.

Koide et al. (1982) showed that 15-HPETE is a potent constrictor of canine basilar artery strips causing a maximal contraction which was 1.5 times greater than that made by serotonin. Further work with highly purified 15-HPETE showed that at a concentration of 20 μM, it causes a contraction of the same arterial preparation which is 71% of that induced by depolarization with 30 mM potassium chloride solution (Takahashi et al., 1985). The contractile effect of 15-HPETE is suggested to be due both to the inhibition of endogenous prostacyclin production and stimulation of further lipoxygenase product release (Koide et al., 1982). This is supported by the finding that isolated mammalian 5-lipoxygenase is activated ten to twentyfold in the presence of HPETEs (Rouzer and Samuelsson, 1986). Asano et al. (1983) showed that 15-HPETE can contract human as well as canine cerebral arteries, but not those from the rabbit. Peripheral blood vessels showed little or no response to the agent. The contraction was resistant to the action of a variety of antagonists and could only be partially blocked by N-ethylmaleimide.

As to the cellular source of HPETEs and HETEs from blood vessels, both the smooth muscle and endothelial layers contribute to their production. The major lipoxygenase product of peripheral artery endothelial cells is 15-HETE (Hopkins et al., 1984; Kühn et al., 1985). Upon incubation with arachidonate, homogenized smooth muscle cells cultured from rabbit aorta were shown to release predominantly 15-HETE, as well as the 12- and 5-HETE isomers by the action of lipoxygenase. Using intact smooth muscle cells cultured from rat aorta, 15-HETE and 11-HETE were shown to be the most abundant

metabolites; their release was attenuated by the cyclooxygenase inhibitors acetylsalicyclic acid and indomethacin (Bailey et al., 1983). The reason for the differences in enzymatic routes of formation of these metabolites is unclear; this could be a reflection of species or methodological differences. The lack of potent inhibitors of the individual lipoxygenases has hampered progress in this area. None are known which can specifically inhibit only one of the 5-, 12-, or 15-lipoxygenase pathways.

Recently a new group of lipoxygenase products have been discovered which are produced by the sequential action of both 15- and 5-lipoxygenases, along with the formation of an intermediate epoxide. The trihydroxyeicosatetraenoic acids which result are called lipoxins (lipoxygenase interaction products), were originally isolated from human leukocytes (Samuelsson et al., 1987). Both 15-HPETE and 15-HETE are precursors for their formation. The two main products, lipoxin A (LXA) and lipoxin B (LXB), possess selective biological properties distinct from those of the leukotrienes. LXA elicits a long-lasting contraction of the guinea pig lung strip, without having any effect on the ileum, and causes arteriolar dilation in the hamster cheek pouch, without effecting plasma leakage (Dahlén et al., 1987). This differentiates it from both 15-HPETE, which causes a weaker, phasic contraction of the lung strip, and the peptidoleukotrienes, which induce plasma leakage from the microvasculature and have marked spasmogenic activity on guinea pig ileum. The effects of the lipoxins on the cerebral vascular bed are unknown to date.

B. Role of cyclooxygenase products in cerebral vasospasm

Cyclooxygenase metabolites of arachidonic acid have long been implicated in the development of cerebral vasospasm. Therapeutically speaking, no course of treatment based on pharmacological manipulation of this pathway has been effective. For a review of the early literature in this area see White (1982).

Cerebral blood vessels have the capacity to synthesize a variety of prostaglandins. Hagen et al. (1979) demonstrated that bovine cerebral arteries could synthesize PGI_2, PGE_2, $PGF_{2\alpha}$, PGD_2, and TXA_2 when incubated with arachidonic acid. Sasaki et al. (1981a) and Maeda et al. (1981) found that PGI_2 was, by far, the major metabolite in canine basilar arteries. In both studies however, TXA_2 was not detected. $PGF_{2\alpha}$ was seen as a minor metabolite, whereas only the latter study showed the presence of small amounts of PGE_2 and PGD_2. Using both arteries and veins obtained from human brain tumour resection, Abdel-Halim et al. (1980) found that the most abundant metabolite was PGI_2, with only a small amount of $PGF_{2\alpha}$ and no TXA_2 measurable. The observation common to all these studies is that the cerebral vascular wall, like those of peripheral arteries, has the greatest capacity to make prostacyclin. Shirahase et al. (1987) showed that dog basilar arteries spontaneously release TXA_2 and they believe that the endothelial cells are the source of this product; no other prostaglandins were investigated. Whether significant amounts of TXA_2 are generated by cerebral arteries is doubtful as Toda et al. (1988) noted that contraction of canine basilar artery by the endoperoxide PGH_2 was not affected by an inhibitor of thromboxane synthetase.

Cerebral arteries contract in response to most prostaglandins (see Cook 1984) except for prostacyclin, which causes vasodilation (Chapleau and White, 1979). The contraction of isolated cerebral arteries in response to blood may be partially due to the formation of cyclooxygenase products. Okamoto et al. (1984) showed that hemolysate from erythrocytes contracted cerebral arteries much more effectively than mesenteric arteries and that the contraction was inhibited by aspirin. Using a perfused circle of Willis from the dog, topically applied blood caused a constriction which was abolished by four different aspirin-like drugs, but not by the thromboxane synthetase inhibitor imidazole (Lang and Maron, 1988). This is in contrast to the study of Okwuasaba et al., (1981b) which showed the contraction of basilar artery ring segments by whole blood was only slightly inhibited by indomethacin.

The hypothesis that cerebral vasospasm could arise from an imbalance between prostacyclin and thromboxane synthesis has received considerable attention (Boullin et al. 1979). In three studies of experimental vasospasm following subarachnoid injection of blood in the dog, the most striking and common feature was that arteries which had been exposed to subarachnoid clot for several days had a diminished capacity to synthesize the vasodilator prostacyclin (Maeda et al., 1981; Sasaki et al., 1981a; Walker et al., 1983). This hypothesis has also been tested using the monkey model of cerebral vasospasm (see Chapter 2). Arteries from the group of animals in which the blood clot had been in place for seven days were less able to synthesize prostacyclin than arteries which had been exposed to blood for only 24

hours. There were no significant differences in thromboxane formation between the arteries from the experimental groups (Nosko et al., 1988). A critique of such studies is that only the basal levels of metabolites are measured from the blood vessel wall itself ex vivo, and one has no idea of the local concentrations of mediators released during the actual course of vasospasm. The loss of prostacyclin generating capacity of arteries which had been in spasm is likely a reflection of the endothelial damage noted in such arteries (Lisczak et al., 1983), and is a plausible contributing factor to the development of vasospasm.

To further investigate the prostaglandin imbalance theory, the level of various prostaglandins in the CSF of patients with subarachnoid hemorrhage has been investigated (Walker et al., 1983; Rodriguez y Baena et al., 1987; Seifert et al., 1987). The levels of prostaglandins were raised in the lumbar CSF of all patients with SAH as opposed to control samples of CSF in each of these studies. Walker et al., (1983) reported that $PGF_{2\alpha}$, PGE_2, and PGI_2 were elevated, but not TXA_2 in five patients with SAH. Seifert et al. (1987) noted that the patients with SAH had elevated CSF levels of both PGI_2 and TXA_2 over samples drawn from non-SAH patients. There was also a correlation between preoperative TXA_2 levels and the amount of blood clot present as shown by CT scan. Patients with SAH showing vasospasm had elevated CSF levels of PGD_2 but not PGI_2 over patients with SAH but no vasospasm (Rodriguez y Baena et al., 1987); PGD_2 and PGI_2 represent vasoconstrictor and vasodilator prostaglandins, respectively. These studies show that there is increased arachidonic

acid metabolism in the subarachnoid space after hemorrhage, with prostaglandins being released and accumulating in the CSF. It is unlikely, however, that the presence of prostaglandins in the CSF accounts for all the facets of vasospasm. At best they are indicators of altered arachidonic acid metabolism during vasospasm.

Studies of vasospasm induced by the subarachnoid injection of blood in the dog have shown some beneficial effect of agents which interfere with the synthesis or action of thromboxane (Sasaki et al., 1982b; Komatsu et al., 1986; Kondo et al., 1987). In each of these models only a single injection of blood was given, which is a less suitable model of late vasospasm. In one study (Komatsu et al., 1986) the course of arterial narrowing was followed for only 24 hours after blood injection, and any beneficial effect of their test drug is truly doubtful at that time. The most recent study by Kondo et al. (1987) using the one-hemorrhage dog model tested the effect of ONO-3708, an antagonist of thromboxane, but this agent was also shown also to block contractions of isolated basilar arteries to $PGF_{2\alpha}$ as well as 15-HPETE, indicating the effect of this drug may include more than just antagonism of thromboxane receptors. All of the drug regimes were started immediately after blood injection, thus none of the agents are likely to resolve delayed clinical vasospasm once it is established. A multicentre clinical trial of the thromboxane synthetase inhibitor OKY-046 showed that it was ineffective in preventing delayed vasospasm (Suzuki et al., 1985), whereas another clinical study did show some beneficial effect of the drug (Yonekawa, 1986). At best the prevention of cerebral vasospasm by means of inhibiting thromboxane formation is controversial.

Prostaglandins and thromboxane are likely not the primary causative agents in the development of cerebral vasospasm, yet results to date suggest some accessory role. Many studies have shown that the formation of prostacyclin in arteries which were in spasm is diminished, which correlates with the endothelial damage often seen in such arteries. Lack of an endogenous vasodilator certainly cannot improve the situation. Whether thromboxane participates in the development of delayed cerebral vasospasm requires further careful study.

C. **Arachidonic acid lipoxygenase metabolites as mediators of cerebral vasospasm**

The study of the role of the lipoxygenase metabolites in cerebral vasospasm is in its infancy. Evidence that a lipoxygenase pathway exists in blood vessels from the peripheral circulation was presented in Section II A. Here I would like to briefly summarize the findings relevant to vasospasm in the cerebrovascular circulation. The papers presented in this thesis deal primarily with providing evidence that a lipoxygenase pathway does exist both in the cerebral blood vessel wall and in cultured smooth muscle and endothelial cells from cerebral vessels. Investigation of the significance of the lipoxygenase pathway in the pathogenesis of cerebral vasospasm is developing along two fronts: what the "classical" leukotrienes of the 5-lipoxygenase pathway, including the peptidoleukotrienes and LTB_4, can do, and what the HPETEs from the various lipoxygenases may bring about in the cerebral circulation.

Contractile effects. Cerebral arteries are known to contract in response to the peptidoleukotrienes in vivo and in vitro (Tagari et al., 1983; Rosenblum, 1985; Jancar et al., 1987 - Chapter 4). Intracarotid injection of LTD_4 in the rat causes a sustained constriction of cerebral arteries (Tagari et al., 1983). The same study showed that isolated human intracranial artery strips respond with a prolonged constriction to LTD_4, although tachyphylaxis occurs very rapidly. The maximal contraction observed was only one third of the force achieved with 5-HT, showing that the leukotrienes have less efficacy than serotonin in constricting cerebral arteries. Interestingly the response to LTD_4 could not be antagonized with FPL 55712, a LTD_4 receptor antagonist (Tagari et al., 1983). Rosenblum (1985) applied LTC_4 topically to mouse cerebral arterioles, causing a contraction which was blocked with FPL 55712. A portion of the results presented in Chapter 4 show that isolated canine basilar artery respond to low concentrations of LTC_4 and LTD_4, although the response is not large in terms of tension development.

In contrast it has been shown that the product of 15-lipoxygenase, 15-HPETE, can constrict canine basilar artery in vitro with similar efficacy as 5-HT and $PGF_{2\alpha}$ (Koide et al., 1982). The contraction was diminished with ETYA (blocks both cyclooxygenase and lipoxygenases) and the calcium-channel blocker verapamil, but not by indomethacin. The authors suggested that the contraction by 15-HPETE was due to the stimulation of further lipoxygenase products from the vascular wall. Comparing cerebral versus coronary arteries from the dog, it has been shown that 15-HPETE caused a marked

contraction of the former and only a weak response in the latter. If the vessels were precontracted with $PGF_{2\alpha}$, coronary vessels relaxed in response to the lipid peroxide whereas cerebral arteries constricted further (Takahashi et al., 1985). The corresponding reduction product, 15-HETE was nearly as potent as 15-HPETE in this preparation.

Source of cerebral lipoxygenase metabolites. Where in the cerebral circulation can the lipoxygenase metabolites arise? Leukotrienes C_4, D_4, and E_4 were isolated from rat brain slices incubated with the calcium ionophore A23187 together with arachidonic acid (Lindgren et al., 1984). Immunohistochemical evidence suggested that nervous tissue and not cerebral blood vessels were the source, although the vascular production of leukotrienes could not be ruled out. In a model of subarachnoid hemorrhage in the gerbil, brains were homogenized after various exposure times to subarachnoid blood and revealed elevated levels of peptidoleukotrienes over saline injected animals (Kiwak et al., 1985). Pooled blood vessels taken from such brains contained 600 ng/g wet weight of slow reacting substance of anaphylaxis activity, whereas grey matter produced 110 ng/g and white matter produced less than 0.2 ng/g. Kiwak and colleagues hypothesized that since blood vessels make up only 2% to 5% of brain wet weight, they must account for only 10% to 27% of leukotriene production seen after SAH. Blood cells such as leukocytes and macrophages may also contribute as they have the capacity to synthesize various lipoxygenase metabolites. Chapter 5 includes evidence that isolated dog cerebral arteries synthesize LTC_4 and LTD_4 when

stimulated with the calcium ionophore A23187. It has already been mentioned that peripheral blood vessels can synthesize leukotrienes with the adventitia having the greatest capacity within the blood vessel wall (Piper and Galton, 1984). A preliminary survey of various arterial sites showed that cerebral arteries could by far release more leukotrienes than renal, coronary and pulmonary arteries (see Piper et al., 1988).

The synthesis of HPETEs and HETEs from the major arteries of the cerebral circulation has not been investigated. Studies using peripheral blood vessels show that they can generate HETEs (Greenwald et al., 1979; Setty and Stuart, 1986). Our observation that canine basilar arteries contract in vitro in response to arachidonic acid (Chapter 4) led us to believe that the cerebral artery wall must be a source of lipoxygenase metabolites (Jancar et al., 1987). The material in Chapter 6 is an investigation of lipoxygenase product formation in bovine cerebral arteries stimulated with arachidonic acid, which shows that these arteries have remarkable ability to produce HPETEs (measured as HETEs), with 15-HETE being the major metabolite. As both endothelial cells and smooth muscle cells from peripheral arteries were shown to have the ability to synthesize HETEs (vide supra), we investigated whether smooth muscle cells which we cultured from bovine cerebral artery could be a source of these products. Chapter 7 reveals that the major lipoxygenase metabolite in these cells is 15-HETE. Constituents of blood are another possible source, the platelet contains only 12-lipoxygenase and could release 12-HETE; leukocytes contain 5- and 15-lipoxygenase activity with eosinophils

showing predominantly the latter, releasing 15-HETE as their major lipoxygenase metabolite (Sigal et al., 1988). The extent to which neuronal tissue contributes to HETE production is not clear. A study by Sautebin et al. (1978) showed the formation of HETE in homogenized brain cortex. Cerebral microvessels may account for the bulk of parenchymal 12-HETE production as Moore et al., (1988a) has shown that they release predominantly this metabolite. It appears that cerebrovascular tissues can readily synthesize HPETEs and HETEs from exogenous arachidonate, whereas a more rigorous stimulation (ie. A23187) results in leukotriene formation.

In vivo studies. It is not known if the subarachnoid injection of leukotriene may cause a delayed arterial narrowing. Sasaki et al., (1981b) produced a condition mimicking cerebral vasospasm in dogs upon injecting 15-HPETE into the cisterna magna. A biphasic contraction was observed using angiography with an initial moderate phase lasting ten hours followed by a more pronounced narrowing two to three days after the injection. The prolonged reduction in vessel diameter persisted until the end of the experiment on the seventh day. The most evident morphological change reported in this study was a marked degeneration of the endothelium. It was reasoned that free radicals generated during clot lysis could be a source of the lipid peroxide 15-HPETE. Yokota et al., (1987) investigated the effect of AA-861, an inhibitor of lipoxygenase, on the development of vasospasm in the dog after double injection of blood into the subarachnoid space. The drug was effective in diminishing the severity of vasospasm development seven days later if the dosage regime was begun shortly

after blood injection, but it did not reverse vasospasm once developed. Although AA-861 is reported to be a selective inhibitor of 5-lipoxygenase (Yoshimoto et al., 1982), we found that the drug effectively inhibited both 15-HETE and 5-HETE release from arachidonic acid stimulated cerebral artery segments (see Chapter 6). In an extension of their previous study, Yokota et al. (1988) measured the levels of peptidoleukotrienes in basilar artery, brain slices and blood clot by incubation with A23187 and arachidonic acid. They found that only brain tissue from animals in the vasospasm group had a slightly elevated capacity to generate peptidoleukotrienes.

Asano et al., (1988) studied which HPETEs could be generated in the CSF, blood clot and basilar artery wall in the two-hemorrhage dog model of cerebral vasospasm. Using HPLC to measure the HETEs which are formed from the reduction of HPETEs, they did not find any in the CSF or in unstimulated basilar artery from animals exposed to SAH, but did find 12-HETE in the blood clot. However if the arterial segments were incubated with A23187 in the presence of arachidonic acid, 5-HETE, LTC_4, and LTB_4 were produced in segments from SAH animals but not from control arteries. The 5-lipoxygenase activity of basilar artery was also shown to be activated in a dose-dependent manner by 15-HPETE but not 15-HETE, which confirms the observation of Rouzer and Samuelsson (1986) with purified enzyme.

Sasaki et al., (1982a) found a number of HPLC peaks with the retention times of HETEs and HPETEs in the analysis of CSF samples from patients with SAH. One peak was postively identified as 5-HETE,

and there was a correlation between the amount of this product as measured in the CSF from ten patients with SAH and the time course of occurrence of cerebral vasospasm. No HETEs or HPETEs were seen in the CSF from patients without SAH.

Most recently a study was presented where the CSF levels of LTC_4 were monitored in patients with SAH. Paoletti et al., (1988) reported that the levels of this product in lumbar CSF were significantly higher from patients with SAH when compared to control samples of CSF. Patients with symptomatic vasospasm had higher levels of LTC_4 than those without vasospasm. Cisternal CSF samples had a sevenfold higher LTC_4 content than those from lumbar CSF. The authors believed that brain tissues accounted for the production of LTC_4 as the white blood cell count from cisternal CSF was less than one cell per ml. The leukotrienes were also shown to be released in episodes of cerebral ischemia and reperfusion (Minamisawa et al., 1988; Saito et al., 1988).

Lipoxygenase hypothesis. It is increasingly apparent that the products of the lipoxygenase arm of the arachidonic acid cascade could play a role in the development of cerebral vasospasm. Several lines of evidence support this theory: 1. both leukotrienes and HPETEs can be synthesized in the brain and both products constrict cerebral arteries, 2. cisternal injection of 15-HPETE caused a condition mimicking delayed cerebral vasospasm in dogs, including damage to endothelium and smooth muscle layers, 3. an inhibitor of both 5- and 15-lipoxygenases, AA-861, decreased the severity of experimental vasospasm induced by subarachnoid injection of blood, 4. the HPETEs

and HETEs inhibit prostacyclin synthetase, diminishing the synthesis of prostacyclin which is a vasodilatory component of vascular tone, 5. small amounts of HPETEs stimulate 5-lipoxygenase activity, representing a synergism between both lipid peroxides and the leukotriene products, and 6. other features documented in cerebral vasospasm, such as the infiltration of cells into the vascular wall and an increase in its permeabilty, myonecrosis and subintimal proliferation of smooth muscle cells are within the spectrum of activities known for the lipoxygenase products. The HPETEs may be generated initially by free radical reactions which occur during the breakdown of the blood clot and degradation of oxyhemoglobin to methemoglobin, with the red blood cell membrane acting as a source of arachidonate (Baker and Loh, 1987). HPETEs could also be generated directly by enzymic activity, as the vascular wall may represent an important additional source of these compounds. However formed, the lipid peroxides act to stimulate 5-lipoxygenase present in neuronal tissue and the vascular wall. Separate from its direct contractile activity, the necrotic damage elicited by 15-HPETE and activated oxygen species which may be present, could account for changes in the vessel wall which reflect the time course of vasospasm and progression to a state where the constriction is not amenable to pharmacological manipulation.

Considered in this light, prevention of vasospasm after subarachnoid hemorrhage still represents a formidable therapeutic challenge. An ideal therapeutic agent must be able to cross the blood-brain barrier and might have to combine anti-lipoxygenase activities with antioxidant effects, without interfering greatly with the

natural processes involved in the removal of the blood clot. Timing would still remain a crucial factor, as drug therapy would have to start as early as possible to defuse processes which lead to later irreversible changes. No one believes that vasospasm is the result of a single agent; the variety of actions and interactions presented by the lipoxygenase-derived eicosanoids explains many facets of this disorder and may eventually lead us to a rational drug therapy of this disease.

III. A Guide to the Questions Asked

The overall aim of this thesis is to investigate arachidonic acid metabolism in cerebral arteries, with regard to the pathogenesis of cerebral vasospasm. The questions asked include:

1. To what extent does the development of experimentally induced vasospasm in the monkey model alter the balance of prostacyclin and thromboxane synthesis by the cerebral artery wall, and what effect does early clot removal have on this?
2. What is the predominant vasomotor action of arachidonic acid on cerebral artery segments in vitro? This is a means to test which of the enzymatic pathways of arachidonic acid metabolism predominates in normal cerebral artery and what the relative contribution of the endothelium is in the production of eicosanoids.
3. Which lipoxygenase metabolites can cerebral arteries in fact synthesize? This has only been investigated to date in arteries from the peripheral circulation and it may indicate whether the cerebral circulation has unique capacities in this regard. This may help to explain why cerebral arteries are prone to vasospasm after SAH.

4. Is it possible to directly investigate the contributions of the smooth muscle and endothelial cells in the production of lipoxygenase metabolites from the cerebral artery wall? This requires the isolation, cultivation, and identification of both cell types from cerebral arteries, which to date has only been attempted for the endothelium.

The first study (Chapter 2) is an investigation of the role cyclooxygenase products of arachidonic acid may play in the development of delayed cerebral vasospasm, using an experimental animal model where clotted blood is placed around the middle cerebral arteries of a monkey (Espinosa et al., 1984). The study was designed to specifically investigate the capacity of the cerebral artery wall to synthesize the vasoconstrictor eicosanoid, thromboxane, and the vasodilator, prostacyclin, after a seven day exposure to a blood clot placed in the subarachnoid space in direct contact with the adventitial wall. In addition, the effect of early clot removal, 24 hours after its placement, was measured. The presence of cerebral vasospasm was assessed with angiography and on the seventh day the arteries from clot placement, sham operated and early clot removal animal groups were harvested and incubated in vitro. The basal release of these two prostanoids into the incubation medium was measured using specific radioimmunoassays. The prostacyclin-thromboxane imbalance hypothesis has not been tested in this model and only some data were available from studies using the canine model and in one study using human tissues.

A pressing issue in the field of vasospasm research is the lack of suitable, simple, and relevant in vitro approaches to the study of this disorder. As mentioned in section I, a most obvious "missing link" is the use of cell culture to understand the basic physiology and pharmacology of the cerebral artery which distinguishes it from arteries found in the periphery. Culture of both endothelial (DeBault et al., 1981) and smooth muscle cells (Diglio et al., 1986) from the cerebral microvasculature has been accomplished, primarily in the pursuit of our understanding of the blood-brain barrier and disorders such as cerebral ischemia. For the study of features relevant to cerebral vasospasm, which also occurs in the large cerebral arteries, cultures from this source would be most appropriate and to date only one reference on the culture of endothelial cells from cerebral artery was published (Goetz et al., 1985). Thus the second major initiative was to develop a simple methodology to isolate and culture both smooth muscle and endothelial cells from cerebral arteries, for the study of their arachidonic acid metabolism (see Chapters 3 and 7). An important facet of this study was to identify the cell lines cultured using both immunocytochemical and electrophysiological criteria. Bovine arteries were chosen as the tissue source due to their availability, large size (which permits the harvest of a maximal number of cells), and the fact that some success had already been reported using this source (Goetz et al., 1985). As well, the culture of cells from bovine aorta is a well established technique (Booyse et al., 1975; Ross et al., 1971) and would allow us to have comparable cell cultures originating from the peripheral circulation for comparison.

The primary pathways of arachidonic acid metabolism in cerebral arteries to products with vasoactive properties is readily determined by investigating their pharmacological responses in vitro. Peripheral arteries respond to stimulation with arachidonic acid with either dilation or constriction, depending upon the vascular bed and the integrity of the endothelium (Singer and Peach, 1983; Miller and Vanhoutte, 1985). The vascular response of canine cerebral arteries to arachidonic acid was tested in an organ bath, and the dependence of these responses on the endothelium was tested by removing it. To assess the mechanism by which arachidonic acid produces its effect, inhibitors of cyclooxygenase and lipoxygenase enzymes, and a leukotriene receptor antagonist were used. In addition, the release of prostacyclin, thromboxane, and peptidoleukotrienes by the stimulation of such segments with arachidonic acid was determined using radioimmunoassay. Canine cerebral artery was chosen since a strong response to arachidonic acid was achieved in this preparation when compared to bovine cerebral artery (unpublished observations). The pharmacology of cerebral arteries is well understood using the canine preparation, and it resembles that of human and monkey cerebral arteries in many aspects (Nosko et al., 1986). Results of this study are presented in Chapter 4.

The production of lipoxygenase metabolites by the cerebrovascular wall is unknown, although it has been suggested that brain leukotrienes originate predominantly from neuronal tissue (Lindgren et al., 1984). The first investigation of lipoxygenase metabolite synthesis by cerebral arteries (Chapter 5) utilized the calcium ionophore A23187,

which is known to release leukotrienes from vascular tissues in the peripheral circulation (Piper et al., 1983; Schulz and Seeger, 1986). High performance liquid chromatography (HPLC) was chosen for analysis, using a method which could separate and detect the leukotrienes of the 5-lipoxygenase pathway (LTB_4, LTC_4, LTD_4, and LTE_4). This study shows the ability of cerebrovascular tissue to synthesize these products in a condition of maximal stimulation.

The second investigation of lipoxygenase product formation (Chapter 6) was borne upon the result of the pharmacological study of the vascular response of isolated cerebral artery to arachidonic acid. Chapter 4 demonstrated that cerebral arteries show a dose-dependent contraction to exogenously added arachidonic acid, which is independent of the presence of the endothelium. The contraction was not affected by inhibitors of cyclooxygenase enzymes but was reduced in the presence of drugs which interfere with the lipoxygenase pathway. Indications were given that lipoxygenase products other than those formed by the 5-lipoxygenase could also be synthesized by the cerebral arterial wall and explain part of the contractile action of arachidonic acid. This includes lipid peroxides such as the HPETEs which are known for their potent contractile action on cerebral artery (Koide et al., 1981; Asano et al., 1983). At the same time a new method was available which allowed the extraction and HPLC analysis of all the lipoxygenase metabolites (both the leukotrienes and the HPETEs, which quickly reduce to HETEs in an aqueous environment and are measured as such) (Eskra et al., 1986).

Thus the biochemical release of lipoxygenase metabolites from cerebral arteries stimulated with arachidonic acid was the focus of the work presented in Chapter 6, giving direct evidence of the chemical nature of the metabolites which may cause the arachidonic-acid induced contraction of cerebral artery. Additionally, using arachidonic acid as a more subtle stimulus than the calcium ionophore A23187, is of more interest in the study of cerebral vasospasm, as the red blood cell membrane in the subarachnoid clot may be a source of this lipid in vivo (Baker and Loh, 1987).

Thus far arachidonic acid metabolism in cerebral arteries has been investigated largely by the use of intact preparations of the vascular wall. The results of Chapter 4 suggested that the endothelium did not contribute in the contractile response elicited by arachidonic acid. An important question is to delineate which of the major cell types of the cerebrovascular wall is responsible for the formation of these lipoxygenase products. With the ability to serially culture smooth muscle cells from bovine cerebral arteries a means was at hand to address this question. Chapter 7 looks at the ability of these cells to synthesize what is the major product of lipoxgenase metabolism in the cerebral artery, 15-HETE. The HPLC method was initially used to see which HETEs are synthesized upon addition of exogenous arachidonic acid. A sensitive and specific radioimmunoassay for 15-HPETE/HETE was employed to investigate details of its enzymatic formation.

The content of these studies is to provide a framework for futher investigation of arachidonic acid metabolism as it applies to the cerebral circulation, particularly in the context of cerebral vasospasm.

The contribution of the prostanoids in the development of this disorder is debatable. The primary thrust of this thesis is to show that the cerebral vascular wall has an active lipoxygenase system, and to lay the groundwork in the cellular nature of the release of its products. Future studies will help to delineate the exact contribution of this eicosanoid pathway in the pathogenesis of cerebral vasospasm.

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CHAPTER 2

EFFECTS OF VASOSPASM ON LEVELS OF PROSTACYCLIN AND THROMBOXANE A_2 IN CEREBRAL ARTERIES OF THE MONKEY

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EFFECTS OF VASOSPASM ON LEVELS OF PROSTACYCLIN AND THROMBOXANE A_2 IN CEREBRAL ARTERIES OF THE MONKEY[1]

Introduction

The prolonged constriction which develops a few days after subarachnoid hemorrhage is a determining factor in the outcome of the hemorrhage. A variety of agents have been suggested to initiate or maintain this vasospasm, but there is evidence that the prostaglandins may play an important contributing role.(3,9,12,26,27) Although many cyclooxygenation products of arachidonic acid are synthesized in cerebral arteries, including prostaglandins $F_{2\alpha}$, D_2 and thromboxane A_2,(10) the predominant product seems to be prostacyclin.(1,18,31) Prostacyclin is a potent dilator of cerebral arteries(12) and is also an inhibitor of platelet aggregation.(36) Thromboxane A_2, however, is a vasoconstrictor that promotes platelet aggregation and thus antagonizes the effects to prostacyclin.(11,24) Whittle and Moncada suggested that control of cellular function may represent an interactive modulation between these two substances.(42) Thus, an imbalance in the relative levels of prostacyclin and thromboxane A_2 may play a role in the development of chronic vasospasm after subarachnoid hemorrhage. Several clinical studies have examined the concentrations of

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[1] A version of this chapter has been published. Nosko, M., Schulz, R., Weir, B., Cook, D.A., and Grace, M. (1988). Neurosurgery 22: 45-50.

arachidonate metabolites in the cerebrospinal fluid (CSF) of subarachnoid hemorrhage patients with and without vasospasm. These have provided further evidence for this theory [8,30,35].

To examine this hypothesis further, we have attempted to determine the basal levels of prostacyclin and thromboxane A_2 in both spastic and nonspastic cerebral arteries of the monkey by measuring their stable metabolites 6-keto prostaglandin $F_{1\alpha}$ and thromboxane B_2, respectively.

Materials and Methods

Twenty-four female cynomolgus monkeys (Macaca fascicularis) were assigned at random to one of three groups designated sham, clot or clot-removal. All animals underwent cerebral angiography for determination of baseline cerebral vessel calibre. Under general anaesthesia, all animals underwent bilateral dissection of the major cerebral vessels from the arachnoid membrane. An average 4.97 g autologous hematoma was placed around the vessels in the subarachnoid spaces in the clot and clot-removal groups. Saline was instilled in the subarachnoid spaces of the sham group. All animals were subjected to a second craniotomy 24 hours after the first procedure. The clot-removal group underwent evacuation of the subarachnoid hematoma. The sham and clot groups were simply reclosed after 3 hours to control for the anaesthetic time. The operative mortality for the combined procedures approached 15%. Animals that failed to survive 24 hours after the first craniotomy were replaced in the study. All animals subjected to the second craniotomy survived. Complete details of the angiography and the operative procedures are

given elsewhere.[25] On day 7 after the subarachnoid hemorrhage, further cerebral angiography was performed. The animals were then killed by exsanguination under general anaesthesia. The cerebral vessels were immediately resected under an operating microscope. The vessels were cleared of connective tissues and blood. A 1 cm section from the proximal portion of each main branch of the circle of Willis (anterior, middle and posterior cerebral arteries; basilar artery) was cut into 1 to 2 mm segments which were placed in a pre-weighed microcentrifuge tube. The tubes were then reweighed to determine the net wet weight of the vessel and were stored at -70°C until processed. Storage times varied from 72 hours to 6 weeks.

Frozen arterial segments were allowed to thaw to room temperature and 1 ml of Ca^{2+}/Mg^{2+}-free phosphate buffered saline (pH 7.4) was added to each tube. The tubes were incubated for 5 minutes at 37°C in a shaking water bath and then centrifuged for 1 min at 8000 x g. The supernatant fluid was discarded. One ml of prewarmed Hanks' balanced salt solution containing 20 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; pH 7.4, Gibco, Burlington, Ontario) was added to each tube and the arterial segments were incubated for 30 minutes at 37°C with gentle agitation and then centrifuged for 1 min at 8000 x g. Aliquots of the supernatant were removed and frozen at -20°C for subsequent radioimmunoassay.

Basal levels of prostacyclin and thromboxane A_2 were measured as their stable hydrolysis products, 6-keto-prostaglandin $F_{1\alpha}$ and thromboxane B_2, respectively, using commercially available radioimmunoassay kits (New England Nuclear, Lachine, Quebec) as

previously described [34]. Each mixture contained 0.1 ml of sample or 5 to 500 pg of an authentic standard, 0.1 ml of diluted antiserum, tritium-labeled thromboxane B_2 or 6-ketoprostaglandin $F_{1\alpha}$, and 50 mM phosphate buffer (pH 7.3 for thromboxane; 6.8 for prostaglandin) containing 0.1% gelatin to a total volume of 0.5 ml. The cross reactivity of the thromboxane B_2 antiserum was 2.0% for prostaglandin D_2 and 0.1% or less prostaglandins E_2, $F_{2\alpha}$, 6-keto-$F_{1\alpha}$ and A_2; for the 6-ketoprostaglandin $F_{1\alpha}$ antiserum, it was 2.5% for prostaglandin E_2 and 0.3% or less for thromboxane B_2 and other prostaglandins. Separate standardization control curves using duplicate serial dilution of standards were constructed for each series of specimens analyzed. Detection of basal leukotriene C_4 levels by radiommunoassay was also attempted, yielding lower than detectable levels with the amount of tissue used for each sample. Activated charcoal suspension was used to separate the free and antibody-bound fractions. The limits of detection for 6-keto prostaglandin $F_{1\alpha}$, thromboxane B_2 and leukotriene C_4 were 10, 5 and 25 pg, respectively.

This protocol was passed by the Animal Ethics Review Committee of the University of Alberta. The care and operation of the animals were performed to the standards of the Canadian Council on Animal Care.

Data Analysis

The radioimmunoassays were performed in a blinded manner. Tissue samples were identified by a random code only, and identification by artery and by treatment group was not revealed until all of the assays had been completed. The concentrations of

6-ketoprostaglandin $F_{1\alpha}$ and thromboxane B_2 were determined as pg/mg wet weight of tissue. Comparisons were made among groups by individual cerebral artery as well as among groups as a composite of all of the arteries. Statistical confidence was obtained using an analysis of variance. The level of significance for all tests of comparison was $p < 0.05$ unless otherwise stated.

Results

Cerebral vasospasm was defined as an angiographically determined reduction of more than 10% in cerebral vessel caliber from the baseline value (pre-subarachnoid hemorrhage caliber). Measurements were made at precisely defined points (25) on each vessel, which approximated the midpoint of the specimen resected for biochemical analysis (proximal 1 cm of each vessel). Vasospasm was not present in any of the sham group animals on day 7 after the subarachnoid hemorrhage.

Severe vasospasm (> 50% calibre reduction) was present in 62% of the clot group animals. Moderate vasospasm (31-50% calibre reduction) was present in 25% of the clot group animals. One animal (13%) demonstrated only mild vasospasm (10-31% calibre reduction). Mild vasospasm was present in 25% of animals in the clot-removal group, with the remainder showing no signs of vasospasm at all.

The vessel calibre of animals from the clot group was significantly reduced from the baseline control value ($p < 0.01$), but no significant difference was found for the clot-removal or sham-operated groups.

The concentrations of thromboxane B_2 and 6-ketoprostaglandin $F_{1\alpha}$ released by the cerebral arteries are given in figures 1 and 2.

There was no statistical difference among the groups (sham, clot, or clot-removal) or the individual vessels (anterior, middle, or posterior cerebral arteries) for levels of thromboxane B_2. Levels of 6-keto-prostaglandin $F_{1\alpha}$ were significantly lower in the clot group as compared to the clot-removal group for the middle cerebral arteries. This difference was also significantly lower ($p < 0.01$) when data for all arteries (clot vs. clot-removal) were combined. No other significant differences were observed.

Radioimmunoassay was also used to estimate the concentration of leukotriene C_4 by these arteries. No measurable leukotriene C_4 release was detected from the arteries in the incubations as performed (detection limit, 25 pg/tube).

Discussion

The opposing actions of prostacyclin and thromboxane A_2 in blood vessels have led to the proposal that these compounds may represent the two arms of a balanced homeostatic control system.[22] Disturbance of the balance between these two substances could lead to the unchecked action of the predominant agent. Indeed, damage to the vascular endothelium has been shown to reduce the synthesis of prostacyclin while other products of the arachidonic acid cascade seem unaffected.[2,4,15] Exposure of canine cerebral vessels to injected subarachnoid blood for 3 to 8 days decreased the synthesis of prostacyclin from control levels, but had no effect on the synthesis of vasoconstrictor prostaglandins.[31] Several studies have evaluated the concentration of vasoconstrictor prostaglandins in the cerebrospinal fluid of normal patients and patients who had suffered aneurysmal

subarachnoid hemorrhage. The general findings are in agreement that the cerebrospinal fluid concentrations of prostaglandins $F_{2\alpha}$ and E_2 and thromboxane B_2 are increased after a subarachnoid hemorrhage.[7,17,38]

Levels of 6-ketoprostaglandin $F_{1\alpha}$ in CSF increased after mock subarachnoid hemorrhage in dogs.[38] In patients with subarachnoid hemorrhage, both an increase[38] and a decrease[8] in CSF prostacyclin levels have been observed; patients in the latter study showed signs of cerebral vasospasm. Tani et al. attempted to prevent vasospasm from developing in subarachnoid hemorrhage patients by inhibiting thromboxane A_2 synthetase. Results were suggestive but not conclusive that postoperative angiographic vasospasm was improved with this approach.[37]

This present study examined the concentrations of prostacyclin and thromboxane A_2 released by the cerebral arteries 7 days after a subarachnoid hemorrhage. No significant difference could be detected among the three experimental groups with respect to thromboxane A_2 production. The clot-removal group, however, demonstrated levels of prostacyclin higher than those of the clot group. The clot group represents animals that had sustained subarachnoid hemorrhages and had developed moderate to severe vasospasm demonstrated by angiography. The clot-removal group represents animals which had sustained subarachnoid hemorrhages, but in which the hematomas were removed; these animals developed, at most, mild vasospasm and 75% had no signs of spasm at all. It thus seems that cerebral vessels in chronic vasospasm may release lower levels of prostacyclin than vessels which were exposed to blood clot for only 24 hours, but such vessels

have comparable levels of thromboxane A_2. Results using canine models of subarachnoid hemorrhage have to date not included data concerning the effect of early clot removal on prostanoid production. However they do show a reduction in prostacyclin production from cerebral arteries subjected to subarachnoid clot for six days, with thromboxane being either not detectable or not significantly altered, as compared to normal arteries.[18,31,38]

Although it seems that prostacyclin levels in the clot-removal group are enhanced over those of sham-operated controls, this does not reach statistical significance. Furthermore, when the clot group is compared with the sham-operated group, there is no statistically significant difference. This observation complicates the interpretation of the data; a possible interpretation is that the observed difference arises from normal production of prostacyclin from spastic arteries and enhanced production from arteries from the clot-removal group. It is also possible that both an enhanced production from clot-removal group arteries and a decreased production from clot group arteries account for the data. It is not possible definitely to determine the answer from the data. Although little is known about the effects of surgical manipulation itself on the production of eicosanoids from blood vessels, it seems at first glance unlikely that the procedure for removal of the clot would increase prostacyclin synthesis. Recent work by Qvarfordt et al., however, has shown that prostacyclin production in regions of arterial stenosis with normal endothelium is increased.[29] Thus, we may speculate that the nonspastic arteries of the clot-removal group may have been stressed in such a way that an increase in prostacyclin

production resulted, whereas the spastic vessels of the clot group suffered endothelial damage, reducing the release of prostacyclin. Further experiments will be required to demonstrate that the endothelium of the vessels in the clot-removal group was intact.

The cellular source of the arachidonic acid metabolites in the blood vessel wall is also relevant because this study involved the harvest of the entire cerebral artery, which is composed of many cell types. The main arachidonic acid metabolite of the peripheral blood vessel is prostacyclin, and the ability to synthesize this product is greatest at the intimal surface, with progressively less being formed toward the adventitia.[23] Indeed, studies with cultured endothelial cells have shown that their major arachidonic acid metabolite is prostacyclin.[19,39] The synthesis of thromboxane is generally associated with platelets and it is possible that thromboxane derived from any platelets adhering to the vessel wall contributes to the thromboxane levels measured, although the preparation was carefully washed. Bovine cerebral arteries can synthesize thromboxane,[10] although this is only a minor product of arachidonic acid metabolism and its cellular source is unclear. Cultured endothelial cells do have a small capacity to synthesize thromboxane,[13] although this is much less than their prostacyclin synthetase activity. The contribution of fibroblasts and any contaminating platelets to the vascular thromboxane release that we observed cannot be excluded, although it seems likely that the smooth muscle cells and endothelium themselves represent the major source of this material.

In vivo laboratory studies have evaluated intravenous infusions of prostacyclin in animals with induced subarachnoid hemorrhage. The effectiveness of this therapy in relieving cerebral vasospasm has been equivocal.[6,28,41] A recent clinical trial of prostacyclin in ischaemic stoke also demonstrated little benefit.[20] Thus, it seems probable that, although a decrease in synthesis of prostacyclin accompanies vasospasm, merely supplying exogenous prostacyclin to vessels in spasm does not reverse the process. It is not surprising prostacyclin generated by cells of the vascular endothelium, which are in intimate contact with the smooth muscle, will have different physiologic effects than the same compound given intravenously.

Although we could not detect any leukotriene release by the spastic or nonspastic arteries, we cannot rule out involvement of these compounds because they are known to be effective in causing contraction of canine cerebral arteries in nanomolar quantities.[14] We recently gave a preliminary account of the release of leukotriene C_4-like activity from isolated canine cerebral artery incubated in the presence of the calcium ionophore A23187 and exogenous arachidonic acid.[33] The participation of other arachidonic acid lipoxygenase metabolites, such as 15-hydroperoxyeicosatetraenoic acid (which can induce delayed vasospasm of the basilar artery in dogs,[32] is a potent constrictor of cerebral arteries in vitro[16] and is known to inhibit prostacyclin production by cultured endothelial cells[21] and blood vessel microsomes[42]), must be evaluated in vasospasm models. In addition to the vasodilatory properties of prostacyclin, the contribution of endothelium-derived relaxation factor in the maintenance

of cerebral circulation remains to be investigated[5] because the effect of this agent is inhibited by hemoglobin, which is released during the process of clot lysis. Thus, any damage to the endothelium seen in vasospasm may contribute to a decreased release of prostacyclin and possibly of other vasorelaxant compounds, resulting in further reduction in blood flow.

The sequence of events that cause vasospasm may involve a process substantially more complex than deficiency of a single compound, and the assignment of one or two biologically active molecules as mediators of chronic cerebral vasospasm is not intended. Persistent vasoconstriction is not likely to be due to a single agent occupying a receptor because desensitization of the tissue to the agonist is an almost universal consequence of this situation. The development of cerebral vasospasm is a complex process, with an interaction among various components of the blood, vessel wall, and subarachnoid space; thus, we can only suggest the involvement of an altered prostacyclin synthesis by cerebral arteries as one facet of its pathogenesis.

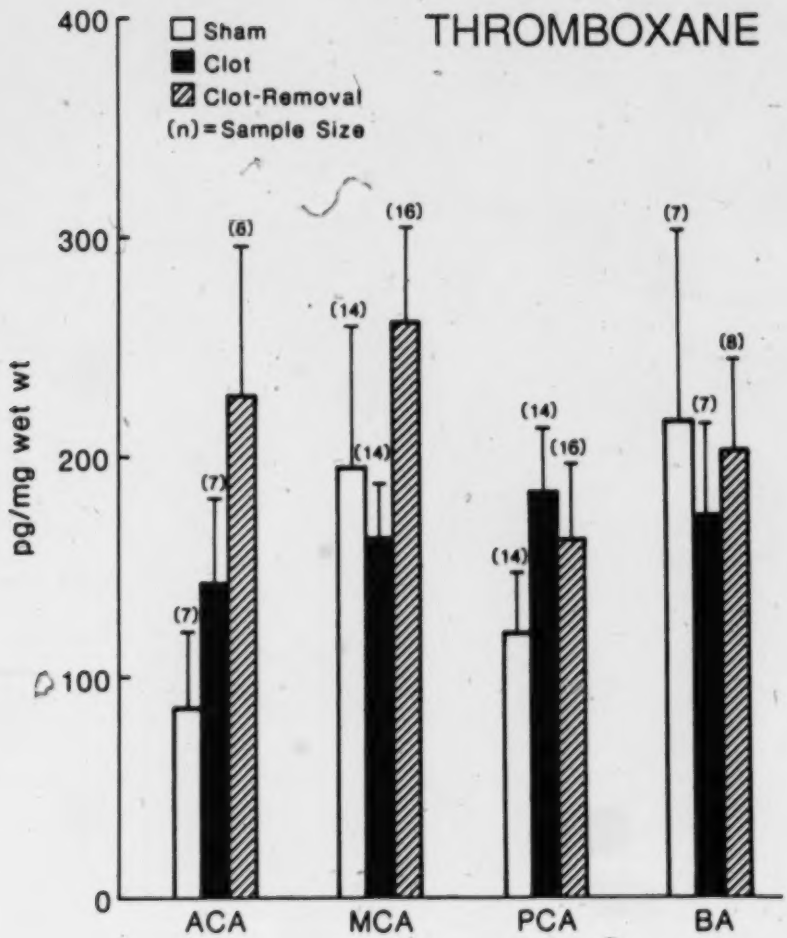

Fig. 1 Basal levels of thromboxane B_2 (metabolite of thromboxane A_2) in monkey cerebral vessels 7 days after subarachnoid hemorrhage induction. ACA, anterior cerebral artery; MCA, middle cerebral artery; PCA, posterior cerebral artery; BA, basilar artery.

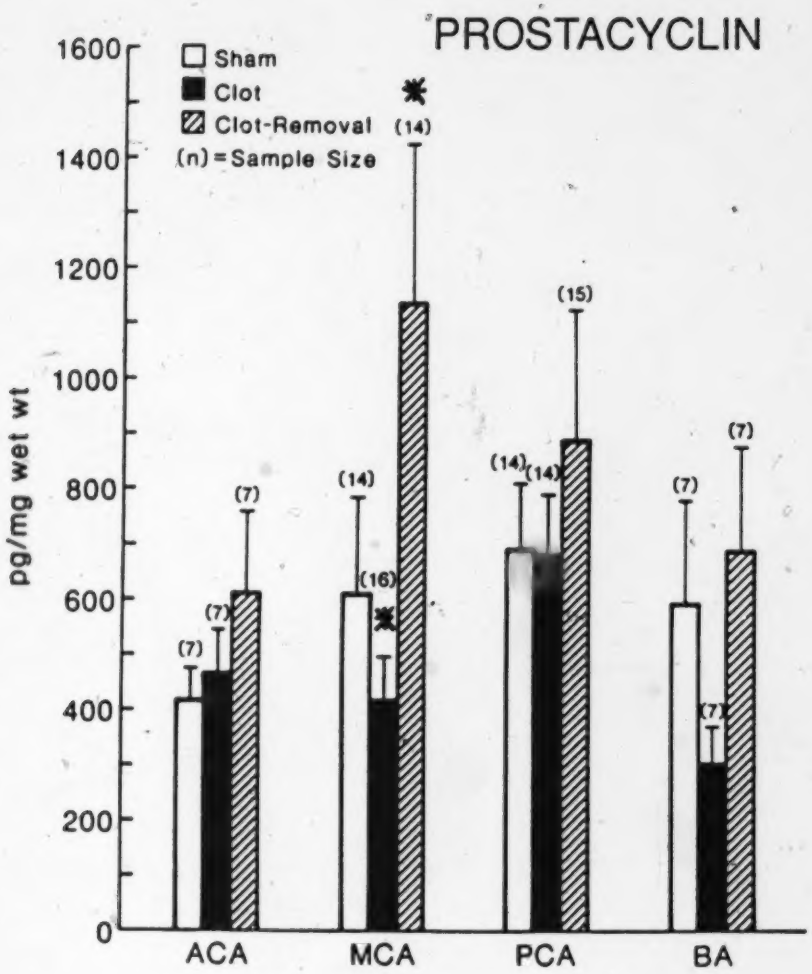

Fig. 2 Basal levels of 6-ketoprostaglandin $F_{1\alpha}$ (metabolite of prostacyclin) in monkey cerebral vessels 7 days after subarachnoid hemorrhage induction. ACA, anterior cerebral artery; MCA, middle cerebral artery; PCA, posterior cerebral artery; BA, basilar artery. * Significant difference ($p<0.01$) between clot and clot-removal groups, for all arteries combined and for the MCA set alone.

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CHAPTER 3

SERIAL CULTURE AND IDENTIFICATION OF SMOOTH MUSCLE AND ENDOTHELIAL CELLS FROM BOVINE CEREBRAL ARTERIES

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Introduction

Establishment of cell culture systems for the investigation of physiological and pathological mechanisms has proved to be an essential addition to studies in whole animals or isolated organs. The ability to examine the function of endothelial and smooth muscle cells from blood vessels has been particularly important in the light of our newly-developed understanding of the important role of the vascular endothelium[15]. Cultures of smooth muscle and endothelial cells from aorta and umbilical vein[3,25,35] have clarified the function of these cell types, however it is clear that responses differ between vascular beds and between large arteries and microvessels. It has been known for many years that cerebral vessels are different from peripheral vessels in terms of anatomy, physiology and pharmacology. The cerebral circulation differs from the peripheral circulation in that the large vessels have a significant resistance role and appear to be the sites at which significant pathological events can develop including migraine and the delayed vasospasm which arises some days after subarachnoid hemorrhage[7]. However, all but one of the reports of cell cultures obtained from blood vessels of the brain deal with the microcirculation. This includes reports on endothelial[8,41] and smooth muscle cells[10] derived from brain microvessels. A single report[18] describes isolation of cerebral arterial endothelium from bovine brain using an enzymic

process to isolate the cells, while there are no reports of the culture of smooth muscle cells from large cerebral arteries.

Cerebrovascular spasm is believed to involve damage to the endothelium and the smooth muscle layer of cerebral arteries. Vasospastic agents are released either from the vessel itself or the clotted blood surrounding it, and we therefore need to examine each cell type separately to determine which agents it could generate under the influence of conditions which approximate to those after subarachnoid hemorrhage. While the use of enzymes in the preparation of primary cell cultures is essential in some systems, we wished to explore the possibility of using a mechanical separation of cells, which would reduce the possible damage to important cell surface markers and obviate the difficulties of variations between batches of enzymes which may complicate the original isolation. Finally, we wished to develop a system which could readily be adapted to isolate smooth muscle or endothelial cells from cerebral arteries of other mammals.

We report here successful establishment of smooth muscle and endothelial cell lines from the large arteries of bovine brain. These cells express specific markers characteristic to their cell type and the smooth muscle cells retain physiologically relevant calcium and potassium conductances, even after multiple passage. This is of considerable importance to the investigation of cerebrovascular disorders and will especially allow a better assessment of the contribution of both cell types in the biology of the cerebral artery wall.

Materials and Methods

Cell isolation and culture. The cell lines described here were cultured from separate sets of pooled cerebral arteries obtained from a total of four to six bovine brains per isolation. Further work in this laboratory has shown that we can isolate viable cultures of smooth muscle and endothelial cells using the arteries from a single brain. The brain was gently dissected free from the opened skull and placed on a clean surface. The cerebral arteries at the base of the brain (Circle of Willis and associated arteries: anterior, middle, posterior and basilar arteries) were removed and placed in ice-cold Krebs bicarbonate buffer (KBB) of the following composition (in mM): NaCl (118), KCl (4.7), $CaCl_2$ (2.5), KH_2PO_4 (1.2), $MgSO_4$ (1.2), $NaHCO_3$ (25), and dextrose (12) containing 300 U/ml of penicillin and 300 μg/ml streptomycin. The preparation was washed twice and transferred to a sterile environment in a laminar flow hood. The arteries were placed in a large Petri dish containing KBB and antibiotics gassed with a mixture of 95% 0_2: 5% CO_2 during the cell isolation. Arteries were then freed of connective tissue and cut into segments 5-10 mm in length. Clotted blood was removed from the lumen of the artery with forceps and the segment placed in a second dish containing fresh oxygenated KBB with antibiotics. These segments were removed individually from the dish, blotted and placed on a sterile surface. The artery was then cut open along the longitudinal axis with fine scissors, rinsed and blotted, and laid flat with the lumen facing upwards. One end of the segment was held in place with forceps while the surface was gently scraped once using a a scalpel with a #15 blade. The cells which had been removed were transferred to a Petri

dish containing 4 ml of isolation medium. The isolation medium used in the primary culture of smooth muscle cells consisted of M199 (Gibco) supplemented with 20% fetal bovine serum (FBS) (Hyclone), 4 mM glutamine, 10 μU/ml insulin, 100 U/ml penicillin-G and 100 μg/ml streptomycin. In our attempts to isolate endothelial cells, we have modified the above isolation medium by adding an equal volume of conditioned medium (M199 with 10% FBS from confluent cultures of bovine aortic endothelial cells) and heparin to a final concentration of 20 U/ml. The cell suspension was aspirated through 18 gauge and 22 gauge needles sequentially to break up larger sheets of cells and placed into a Petri dish, 35 mm in diameter, or in a 25 cm^2 flask. Cells were incubated at 37°C in humidified air containing 5% CO_2.

Cultures were not disturbed for one week, after which two-thirds of the media was carefully removed and replaced with fresh isolation medium. Thereafter each week a complete exchange of medium was provided. Cells which had attached and firmly spread on the surface of the dish were not usually seen until 9 to 14 days after the initial isolation, at which point both single cells and small colonies of cells were seen.

When the primary cultures were almost confluent, the medium was replaced with culture medium (M199, 20% FBS, 4mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin). Cells were scraped off of the dish with a rubber policeman and triturated using a narrow-bore 5 ml pipette before transferring them to a 75 cm^2 flask. Cells were routinely subcultured in this manner at a split ratio of 1:2 or 1:3, and fed once a week.

Other cells in this study included bovine aortic endothelial cells isolated and cultured without the use of enzymes[37]. Cells were grown in M199 with 10% FBS, 4mM glutamine and antibiotics. The smooth muscle cell line derived from rat thoracic aorta, A7r5[27], was purchased from the American Type Culture Collection, Rockville, MD, (ATCC-CRL 1444) and was maintained in Dulbecco's minimal essential medium with 10% FBS and antibiotics. This cell line was also passaged as described.

All cultures were free of mycoplasma contamination using a direct agar culture method[29] as tested by Dr. Janet Robertson, Department of Medical Microbiology, University of Alberta.

Preparation of cells for immunocytochemistry - cell-spot method. Cells grown in 75 cm^2 flasks at various passage numbers, either pre- or post-confluent, were scraped up into 3 to 5 ml of cold culture medium, triturated with a 5 ml pipette to disperse the cells and kept on ice. One drop of the suspension was placed onto a glass slide. Cells were air-dried at 37°C for 30 min, fixed in ethanol : acetone (1:1) for 3 min at 4°C and washed for five minutes on a rotating platform in three changes of cold saline.

Immunocytochemistry. An indirect immunoperoxidase technique[31,42] was used to test all cultures derived from bovine cerebral artery. Bovine aortic endothelial cells and A7r5 smooth muscle cells served as controls. The following primary antibodies were used: rabbit anti-human Factor VIII associated protein (Behring-Hoechst, Montreal, Que.), mouse monoclonal anti-smooth muscle

α-actin[40] (Sigma, St. Louis, MO) and rabbit anti-chicken desmin (ICN, Montreal, Que.).

Whole bovine serum (Sigma) diluted 1:25 in Tris buffer containing 15mM NaN_3 and 1% bovine serum albumin was added to coat the spot of cells. Excess fluid was drained off and either the primary antibody or normal serum as a negative control was added. Mouse serum was used in place of the primary antibody in the control for the assay of smooth muscle α-actin and rabbit serum was used as a control for the Factor VIII associated protein and desmin assays. The sample was incubated for 1 hr in a humid chamber at 37°C. The slides were washed in three changes of cold saline as described, and then incubated with the appropriate link reagent diluted in Tris buffer for 30 min at 37°C. The link reagents used and their dilutions were as follows: rabbit anti-mouse Ig (1:150) and goat anti-rabbit IgG (1:100) (Dako-Cedarlane, Hornby, Ont.).

Slides were washed and the matching peroxidase-anti-peroxidase (PAP) complex was added and incubated for a further 30 min at 4°C. The PAP complexes used included: mouse PAP (1:150) (Sternberger-Meyer, Jarrettsville, MD) and rabbit PAP (1:300) (Sigma).

After washing the slides, the immunolocalized peroxidase enzymes were visualized by exposure to Graham and Karnovsky medium (0.7% diaminobenzidine and 0.03% H_2O_2 in Tris buffer) for 8 min at room temperature[19]. The slides were washed under running tap water, counterstained with hematoxylin, dehydrated in a series of graded ethanol solutions, and cleared in xylene. Coverslips were then mounted.

Staining for lectin binding sites. The above procedure was modified for the localization of lectin binding proteins[23]. Lectin-H (Dade, Miami, FL), a plant agglutinin extracted from Ulex europaeus which has specificity for α-L-fucose residues, was diluted 1:2 in Tris buffer and added to the cell spot on the slide after the cells had been air dried, fixed and washed as described. The sample was incubated for 1 hr at 37°C followed by a further hour at room temperature. The slides were then washed for 5 min with 0.01% Triton-X in saline. Whole bovine serum (1:25) was added to coat the spot followed by either goat anti-Ulex europaeus agglutinin I (Vector Labs via Cedarlane Labs, Hornby, Ont.) or normal goat serum as a negative control. The sample was incubated for 16 hr at 4°C and the lectin binding sites were localized as described above. Rabbit anti-goat IgG (1:150) was used as the link reagent and goat PAP (1:175) was used as the developing reagent (both from Dako-Cedarlane, Hornby, Ont.). As a further control, the specific lectin binding was inhibited by pretreatment of the lectin dilution with 0.2 M α-L-fucose (Sigma) in Tris buffer.

Measurement of whole-cell currents. For electrophysiological experiments, cerebral artery smooth muscle cells from confluent stocks of serially passaged cultures were plated at a low density in 35 mm Corning tissue culture dishes and were used within 4 hours. Spherical cells with diameters of less than 30 μm were selected for recording. Whole-cell currents were recorded following the procedure of Hamill et al.[20] using an Axon Instruments patch-clamp amplifier. The patch pipettes had tip resistances of 1-4 MΩ and the input

resistance of the cells was 5-10 GΩ. Data were only accepted for analysis if the product of the series resistance and membrane current was less than 5 mV. Series resistance compensation was not employed as this restriction could generally be met. Leakage conductance was determined from small, hyperpolarizing voltage steps and was used to correct the current records for leakage currents. The bath solution used in experiments to determine total membrane currents was (mM): NaCl (130), KCl (5.4), $CaCl_2$ (1.8), $MgCl_2$ (1.2), HEPES (10) and glucose (5.2). The pipette solution was (mM): KCl (131.7), $MgCl_2$ (1.2), EGTA (2), HEPES (10) and glucose (10). In experiments involving blockade of potassium currents the pipette solution was (mM): $CsCl_2$ (121.7), tetraethylammonium chloride (TEA) (10), $MgCl_2$ (1.2), EGTA (2), HEPES (10) and glucose (10). The dihydropyridine, Bay K 8644, was kindly provided by Miles Laboratories (Dr. Scriabine, New Haven, CT) and was prepared as a 10 mM stock solution in ethanol. The final concentration of solvent was less than 0.01% and had no effect on the calcium currents.

Results

Cell Culture. Examination of the medium containing the cells scraped from the lumen of the cerebral arteries revealed small sheets and strands of cells, with approximately 5-50 cells per fragment. No attachment of the fragments was seen until 9-14 days after the initial isolation. Use of human fibronectin, gelatin, laminin or Type IV collagen to coat the dishes did not improve the plating efficiency of the primary cell cultures.

The majority of isolations led to primary cultures showing a predominance of cells which were smooth muscle-like in appearance. The cells were thin and spindle-shaped, with cellular overlap and no obvious local organization. Primary cultures at least four weeks old showing greater than two-thirds confluency were subcultured by scraping cells into culture medium and dividing them into new dishes at a ratio of 1:3. After the first passage the cultures appeared not to be contaminated with endothelial cells. Smooth muscle cells of the three strains characterized in this study (BCA 16-18) showed similar morphology and growth characteristics; BCA 16 is shown in Fig 1a. Confluent cultures showed a layered "hill and valley" morphology typical of smooth muscle cells. Cell density of cultures in their fourth passage ranged from 1.9 - 6.4 X 10^4 cells/cm^2. More recently, we have obtained cultures with similar morphology from cerebral vessels of dog and monkey.

When the isolation medium contained both heparin and medium conditioned with bovine aortic endothelial cells, some primary cultures showed patches of closely opposed polygonal cells of endothelial morphology. In two out of three isolation attempts using these culture conditions, the majority of cells in primary culture had endothelial morphology with the other cells appearing as smooth muscle cells. These cells grew slowly and reached confluence in 4-5 weeks. One such culture, BCA 20, after its subculture showed no contamination by smooth muscle cells and is shown in fourth passage in Fig. 1b. Cell density of confluent and post-confluent cultures was 8.4 x 10^4 and 16 x 10^4 cells/cm^2, respectively. It is substantially more difficult to

establish endothelial cell cultures from cerebral vessels. We have also attempted to obtain cerebrovascular endothelial cells by the method of Goetz et al.[18], in which collagenase is perfused through cerebral artery segments, but we were not successful in obtaining viable cell cultures by this means.

Immunocytochemistry. The overall staining characteristics of the cell cultures tested is shown in Table 1. Cells cultured from frozen stocks showed no alterations in their staining patterns.

Smooth muscle α-actin. All the smooth muscle cultures including the A7r5 cells and those from bovine cerebral arteries (BCA 16-18) showed positive immunoperoxidase staining with a monoclonal antibody which recognizes only the smooth muscle α-isoform of actin[40] (Fig. 2a, left). Staining was seen in the cytoplasm and was not observed in the nucleus. The appropriate control where mouse serum is incubated with the cells in place of the primary antibody is seen in the right of Figure 2a. In contrast, the endothelial cultures examined in this study showed a complete lack of staining for this marker (data not shown).

Desmin. Our initial intention was to distinguish smooth muscle from endothelial cell cultures by staining for the intermediate filament desmin, a marker generally associated with myogenic differentiation[28]. However, it was found to stain all cell cultures in this study, including endothelial cell cultures from both aorta and cerebral artery. The specific immunoperoxidase reaction product was localized to the cytoplasm with a granular appearance as shown for smooth muscle cells (Fig. 2b) and endothelial cells (Fig. 3c) from cerebral arteries.

Factor VIII-related antigen. Both the control aortic endothelial cells (BA 3) and endothelial cells cultured from cerebral artery (BCA 20) exhibited positive immunoperoxidase staining with antiserum to Factor VIII-related antigen. Often a halo-like staining of the perinuclear region of the cell was seen as shown for BCA 20 in Fig. 3a. Intensity of staining varied from cell to cell but nearly all cells showed some degree of specific staining as compared to control (Fig. 3a). In contrast none of the other smooth muscle cell cultures (BCA 16-18) or the A7r5 control cells stained with this antiserum (data not shown).

Lectin. Specific staining for lectin binding sites was observed in both aortic and cerebrovascular endothelial cells as shown for BCA 20 in Fig. 3b. Specific staining was abolished by two means: 1. incubation with normal goat serum instead of the anti-lectin agglutinin and 2. preincubation of the lectin extract with a solution of 0.2 M α-L-fucose[23]. Endothelial cells showed strong homogenous staining of the cytoplasm which was more diffuse than that observed with Factor VIII-related antiserum. Individual cells showed varying degrees of staining intensity yet were clearly distinguishable from control preparations (Fig. 3b). The control A7r5 smooth-muscle cell line and the cerebrovascular smooth muscle cell cultures showed a moderately high background staining which was not distinguishable from their control preparations incubated with normal goat serum (data not shown).

Calcium and potassium currents. When the smooth muscle cells were bathed in a physiological solution and the patch pipette contained

a high potassium solution, depolarizing voltage steps from a holding potential of -65 mV elicited a time-dependent inward current followed by an outward current (Fig. 4a). Inward and outward current components were separated and the ion channels carrying the currents were identified by substitution of impermeant ions for permeant ones and by the application of pharmacological antagonists. The outward currents were found to be carried by potassium ions (Fig. 4c) as they were absent when the pipette solution contained TEA, a potassium channel blocker[21] and cesium, an impermeant ion, in place of potassium (Fig. 4b). The outward currents were also reversibly suppressed by external application of TEA (Fig. 4d). When the potassium currents had been blocked, the remaining inward currents were identified as calcium currents (Fig. 4b) in that they were reversibly blocked by lanthanum which is an ion known to block calcium channels[21] (Fig. 4d) and were increased 2-3 fold by the calcium channel agonist, Bay K 8644. Further experiments would be required to determine whether there are multiple types of calcium channels as has been described for numerous other cell types. Since all inward currents were completely blocked by lanthanum, there are apparently no inward sodium currents in these cells.

Discussion

Our study provides evidence for the successful isolation and subculture of both smooth muscle and endothelial cells from bovine cerebral arteries, without the use of enzymes. The cell cultures were identified on the basis of morphology, growth behaviour, immunocytochemical criteria, and electrophysiological properties.

This is the first report of the culture of smooth muscle cells from large cerebral arteries. The cells are robust, proliferate rapidly and show the expression of smooth muscle α-actin, even after repeated subculture and frozen storage. This particular isoactin has been shown to be present only in smooth muscle[46]. This has been confirmed by the use of a monoclonal antibody raised against a synthetic decapeptide identical in sequence to the amino terminus of smooth muscle α-actin[40]. Two established clonal smooth muscle cell lines have also been shown to express this smooth muscle marker[12,43]. The presence of this actin isoform was seen in cultures of brain microvascular smooth muscle cells[10,30], although one study[10] showed a loss of this marker in late-passage cultures. It is notable that the clonal A7r5 cell line from rat aorta[27] has not previously been shown to possess this marker of smooth muscle differentiation.

Investigation of actin content and isoform expression in relation to growth and cytodifferentiation of smooth muscle cells both in vivo and in vitro[2,33,34] has added a further issue to the discussion about differences between cultured smooth muscle cells and those found in the vascular wall. It has been proposed that there is a continuous spectrum of phenotypic expression in smooth muscle cells from a "contractile" to a "synthetic" state[4]. The loss of smooth muscle α-actin is not a prerequisite for the growth of vascular smooth muscle cells in vitro[33], although a three-fold higher α-actin content was observed in post-confluent cells as compared to those in their log phase of growth. Rat aortic smooth muscle cells showed a decrease in smooth muscle α-actin content from cells in primary culture to those in

their fifth passage[40], but evidence was also provided, in agreement with other studies using multiply passaged cells, confirming that a small but significant amount of the isoform remains[2,12,43]. We have observed no decrease in the intensity of α-actin staining in our cells after several passages, and it is thus possible that the changes seen by others arise from the use of enzymes during isolation and subculture.

The smooth muscle cells cultured from cerebral artery stain positively with the antibody to desmin. Although the intermediary filament composition of cerebral artery smooth muscle cells is unknown, it has been shown that in vascular smooth muscle obtained from the peripheral circulation, vimentin is the major intermediate filament protein while visceral smooth muscle contains primarily desmin[16]. Further studies have shown that there is a definite heterogeneity of smooth muscle cells in terms of their intermediate filament subtype and distribution in the various layers of the aortic blood vessel wall[38,45]. Although the majority of cells are vimentin-positive, there is a distinct subset which show the presence of both desmin and vimentin, and those cells containing desmin are closer to the vessel lumen. It remains to be seen whether this vascular smooth muscle heterogeneity also exists in cerebral artery and, if so, whether this has any bearing on the functional differences between peripheral and cerebral arteries.

Voltage-clamp analysis of membrane ionic currents from cerebrovascular smooth muscle cells revealed that the cells express voltage-dependent calcium and potassium channels and lack voltage-dependent sodium channels. This profile of channel types is

characteristic of vascular smooth muscle [22,47] and thus demonstrates that these properties were not changed with serial passage. It is extremely difficult to conduct voltage-clamp experiments on vascular smooth muscle cells in intact arteries because of the technical difficulties in obtaining adequate control of the membrane potential.[22] Since the smooth muscle cells grown in tissue culture can be effectively voltage-clamped and reproducibly cultured, they make a useful system in which to study the ion channels of vascular smooth muscle. Of particular interest is the pharmacology of the calcium channels since organic calcium channel antagonists are widely used in the management of such conditions as cerebrovascular spasm.

The single previous description of the culture of cerebral artery endothelial cells from bovine brain is given by Goetz et al.[18]. They isolated cells by use of collagenase while, in contrast, we have isolated endothelial cells by a gently scraping of the lumen of the arteries. Freshly plated cultures contained sheets and strands of cells remarkably similar to those reported previously using enzyme perfusion[18]. We cannot, however, consistently isolate pure cultures of endothelium. The majority of our isolation attempts, regardless of the culture medium used, have resulted in smooth muscle cell cultures. In our hands the methods of Goetz et al.[18] did not afford viable cultures of cerebral artery endothelium. We believe that the key to improving the reproducibility of our technique lies mostly in the selective removal of the endothelium from the wall of the cerebral artery such that the ratio of endothelial cells to smooth muscle cells in the primary isolate is maximal.

Medium conditioned by endothelial cells is known to contain factors some of which stimulate and some of which inhibit smooth muscle proliferation[4]. The inhibitory factors, which may include heparin itself[5,6], seem to predominate over the stimulatory factors when confluent endothelial cells are used to condition the medium. The medium may also contain autocrine factors for endothelial cell growth as shown for capillary endothelial cells[39] and for aortic endothelial cells[17]. Heparin is also known to stimulate the growth of endothelial cells[44]. It was demonstrated that heparin, at a concentration of 100 μg/ml, inhibited the growth of cerebromicrovascular smooth muscle cells in their first three days of growth[10]. Since either scraping or enzymatic digestion of the arterial lumen will often result in a primary culture containing more than one cell type, some selection of one type over the other is necessary, either by use of special media, selective subculture techniques[36], cloning, or by substrate modification such as a substratum of purified fibrinogen which allows attachment of endothelial but not smooth muscle cells[9].

The homogenous cultures of endothelial cells obtained stained positively with the antibody to Factor VIII-related antigen, which remains the most reliable marker of endothelium in tissues[24] as well as in cultured endothelial cells.[25] However the lack of expression of Factor VIII-related antigen in some vascular endothelia such as cells from the renal vasculature prompted the development of lectins such as that from Ulex europaeus (UEA-I) as markers for vascular endothelium[23]. This lectin has a specificity for α-L-fucose-containing glycocompounds and binds exclusively to the endothelium in all human

tissues studied as well as in cultured endothelial cells. Using double immunoflorescent labelling experiments, a distinct staining pattern for Factor VIII-related antigen and UEA-I was shown within the cells[23]. We found that the endothelial cell cultures from both cerebral artery and aorta, but none of the smooth muscle cell cultures specifically bound UEA-I. Although it has been reported that UEA-I stains only human endothelium in tissue sections from human and animal sources[1] recent studies give clear evidence that endothelial cells cultured from rat venules and aorta[32] and bovine lymph vessels[26] do indeed bind UEA-I. We believe that when used in conjunction with more specific markers of endothelial cells, lectin binding is a useful criterion for their identification.

The cerebral artery endothelial cultures also stained for desmin. Endothelial cells from human umbilical vein were shown to contain vimentin but not desmin[11]. A recent systematic study by Fujimoto and Singer[14] clearly indicated that endothelial cells in certain capillary beds of the chicken contain either desmin alone or both desmin and vimentin whereas the endothelia of all large blood vessels examined contained only vimentin. The endothelium of brain capillaries stained only for vimentin, but no data was provided for larger cerebral arteries. It is clear that further characterization of the intermediate filament type in both endothelial and smooth muscle cells of mammalian cerebral arteries is desirable.

The importance of the establishment of these cell lines to an investigation of the mechanism of cerebrovascular disease is considerable. Cerebrovascular spasm secondary to subarachnoid

hemorrhage may involve changes in the function of endothelium, smooth muscle cells, or both. A recent study of experimental vasospasm showed that oxyhemoglobin caused a progressive contraction and myonecrosis of aortic smooth muscle cells in culture, over a seven-day period[13]. Peripheral blood vessels are quite different pharmacologically and although cultures of both cell types have been well documented for peripheral vessels, such cultures may be a poor model for cerebral blood vessels. The ability of the endothelium or the smooth muscle cells to generate potential spasmogenic compounds has been subject to considerable indirect investigation using intact vessels, but it seems probable that definitive answers can best be supplied from studies of single cell types in culture.

Fig. 1 A. Phase-contrast micrograph of confluent smooth muscle cell culture from bovine cerebral arteries (BCA 16) in seventh passage. Scale bar 40 μm.

B. Phase-contrast micrograph of confluent endothelial cell culture from bovine cerebral arteries (BCA 20) in fourth passage.

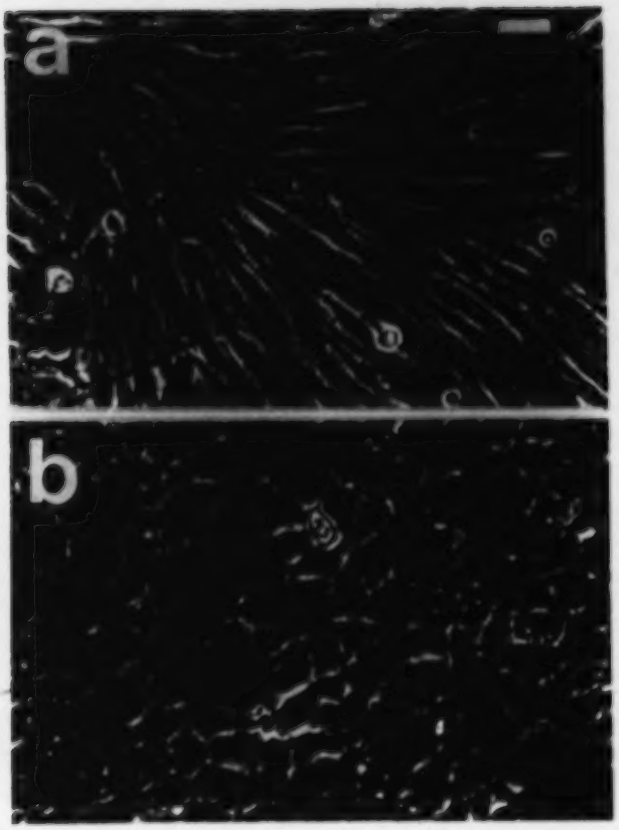

a
b

Fig. 2 Immunocytochemistry of bovine cerebral artery smooth muscle cells (BCA 18) in sixth passage using indirect immunoperoxidase technique. Cell nuclei counterstained with hematoxylin. Scale bar 20 μm.

A. Left: Positive staining with antiserum to smooth muscle α-actin.

Right: Control using mouse serum in place of primary antibody.

B. Left: Positive staining with antiserum to desmin.

Right: Control using rabbit serum.

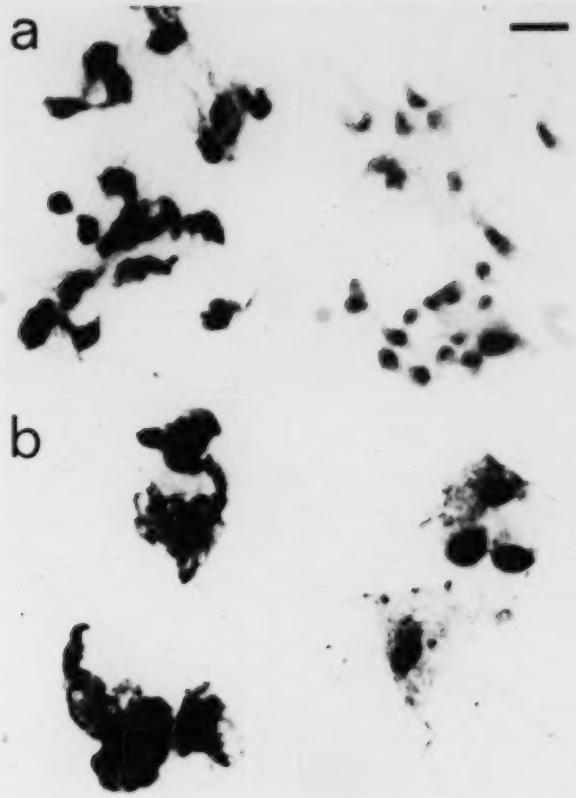
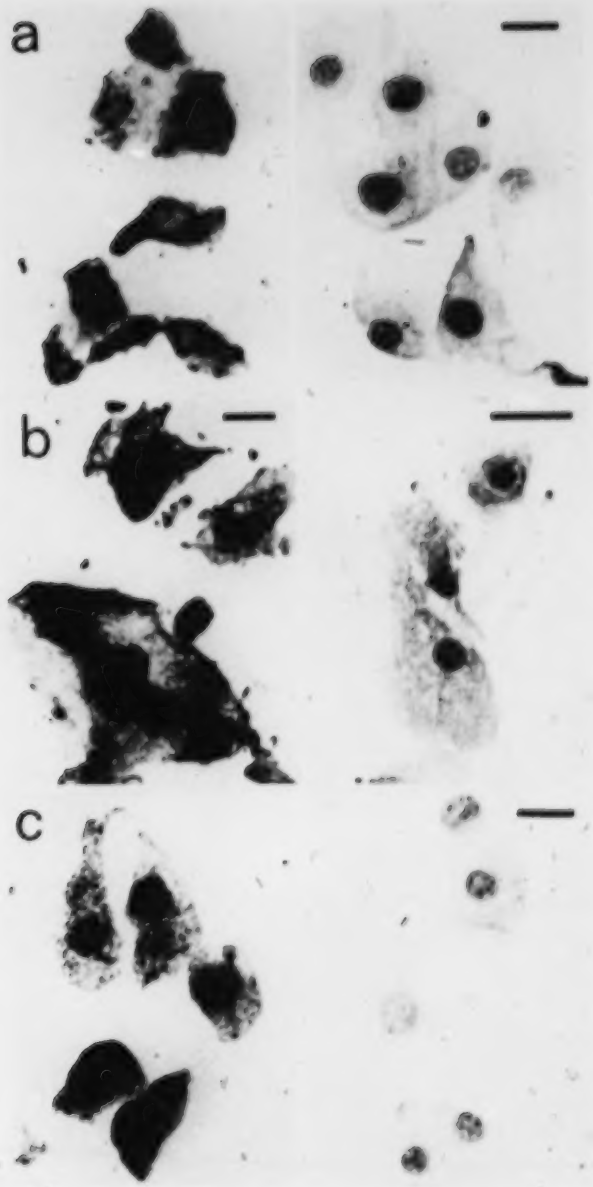

a
b

Fig. 3 Immunocytochemistry of bovine cerebral artery endothelial cells (BCA 20) in fourth passages using indirect immunoperoxidase technique. Cell nuclei counterstained with hematoxylin. Scale bar 20 μm.

A. Left: Positive staining with antiserum to Factor VIII-related antigen. Note granular staining of the perinuclear cytoplasm.
Right: Control using rabbit serum in place of primary antibody.

B. Left: Positive staining with antiserum to UEA-I following incubation with an extract of Ulex europaeus.
Right: Control using goat serum.

C. Left: Positive staining with antiserum to desmin.
Right: Control using rabbit serum.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c

Fig. 4 Membrane ionic currents measured in voltage-clamped smooth muscle cells from cerebral arteries (BCA 17). Current traces (A-C) are for voltage steps to +5 mV from a holding potential of -65 mV. A and C were recorded from the same cell.

A. Inward current (downward deflection) followed by an outward current elicited by a depolarizing voltage step (V) shown at top. The pipette solution (intracellular solution) contained 132 mM K^+.

B. Inward calcium current observed when potassium currents are blocked. Pipette solution contained 122 mM Cs^{2+} and 10 mM TEA.

C. Outward current carried by potassium. Pipette solution contained 132 mM K^+ and 10 μM La^{3+} was added to the bath solution.

D. Steady-state potassium currents (•) and peak inward calcium currents (■) plotted against the test voltage applied across the cell membrane. Potassium currents were recorded in the presence of 10 μM La^{3+} in the bath solution and were suppressed by addition of 5 mM TEA to the bath solution (o). Calcium currents were blocked by addition of 10 μM La^{3+} to the bath solution (□).

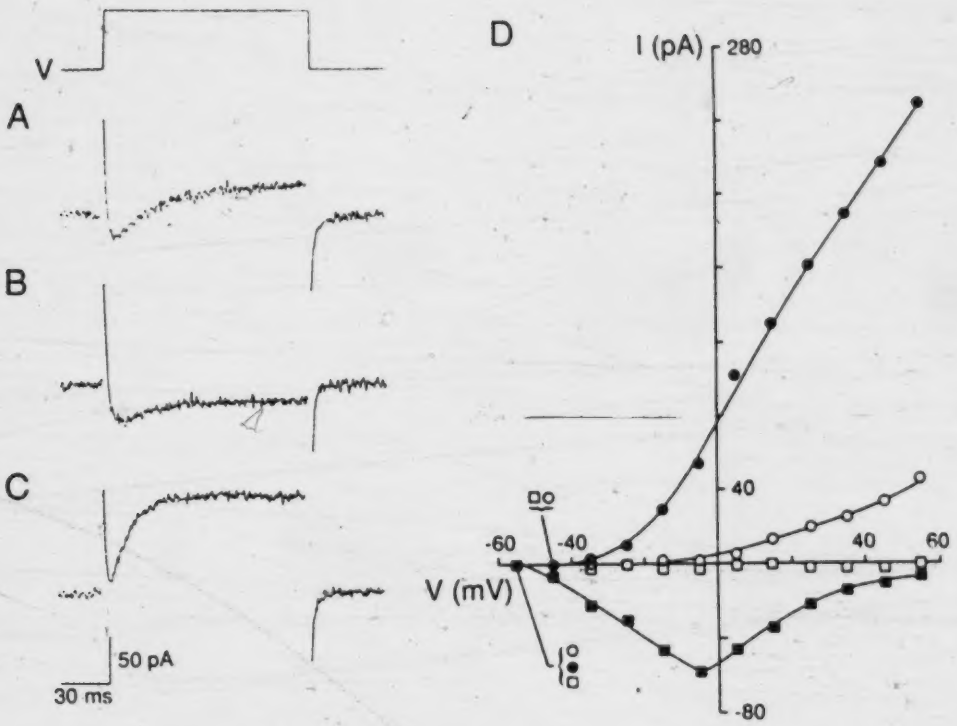
V
A
B
C
50 pA
30 ms
D
I (pA)
280
40
-80
-60
-40
40
60
V (mV)

Table 1 Summary of Immunocytochemistry

Culture	Passage Tested	Cell Type	Marker: Factor VIII	UEA-I	α-actin	desmin
BA 3	3-5	endothelial	+	+	-	+
BCA 20	3-5	endothelial	+	+	-	+
A7r5#	unknown	smooth muscle	-	-	+	+
BCA 16	5-7	smooth muscle	-	-	+	+
BCA 17	5-7	smooth muscle	-	-	+	+
BCA 18	5-7	smooth muscle	-	-	+	+

smooth muscle cell line from rat aorta (ATCC-CRL 1444)

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CHAPTER 4

MECHANISMS OF ARACHIDONIC ACID - INDUCED CONTRACTIONS OF CANINE CEREBRAL ARTERIES

CHAPTER 4

MECHANISMS OF ARACHIDONIC ACID - INDUCED CONTRACTIONS OF CANINE CEREBRAL ARTERIES[1]

Introduction

Isolated peripheral blood vessels are known to respond to exogenous arachidonic acid either by contraction or by relaxation depending on the vessel in question and the dose of arachidonic acid. In general the responses require the presence of an intact endothelium and appear to result from metabolism of arachidonic acid by the cyclooxygenase pathway to give vasoactive products (DeMay and Vanhoutte, 1982). The situation in cerebral arteries is more controversial. Koide et al. (1981) reported that arachidonic acid causes contractions of canine basilar artery which are not affected by cyclooxygenase inhibitors. More recently Fujiwara et al. (1986) have reported that in helical strips of the same preparation the contractile response is abolished by indomethacin and mechanical rubbing of the intima while thromboxane levels are somewhat reduced by these procedures although only to about 50% of the control values.

It is clear that in addition to any actions of its own, arachidonic acid can provide substrate for the cyclooxygenase pathway leading to prostaglandins, prostacyclin and thromboxane, or for the lipoxygenase

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pathway leading to hydroxy- and hydroperoxy-eicosatetraenoic acids and leukotrienes. Both pathways thus produce compounds with intense vasoactive properties. In cerebral arteries it is well established that cyclooxygenase products can be synthesized leading to constriction (thromboxane and some prostaglandins) or dilation (prostacyclin) (Hagen et al. 1979). Indeed some pathological conditions, such as the prolonged constriction of cerebral arteries which often follows subarachnoid hemorrhage, may arise from imbalances in this system (Boullin et al. 1979). We have recently reported preliminary studies in which cerebral arterial segments stimulated with the calcium ionophore A23187 were shown to release leukotrienes C_4 and D_4 (Schulz et al., 1986). It thus seems that a variety of vasoactive products can be produced from arachidonic acid in the cerebral vasculature, by the endothelial cells, the smooth muscle, or even by other components of the vessel wall.

In order to clarify these questions we have investigated the arachidonic acid-induced contractions of ring preparations of canine cerebral arteries using inhibitors of arachidonic acid metabolism. We have also examined the production of thromboxane, prostacyclin and leukotrienes by this same preparation.

Materials and Methods

Studies of contractility. Adult dogs of either sex were killed with an overdose of pentobarbital (60 mg/kg) and the brain was rapidly removed. The basilar and middle cerebral arteries were dissected free of surrounding tissue and cut into rings 3-4 mm wide

These rings were suspended under a resting tension of 1 g in organ baths of 10 ml working volume. The bathing medium used was a Krebs bicarbonate solution of the following composition in mM: Na^+ 132, K^+ 5.9, Ca^{2+} 2.5, Mg^{2+} 1.2, Cl^- 122.7, HCO_3^- 25, SO_4^{2-} 1.2, H_2PO4 1.2 and dextrose 11, gassed with 95% oxygen: 5% carbon dioxide. Where the procedure called for removal of the endothelium, this was carried out by gentle friction with a stainless steel wire. After a 90 min equilibration period, responses were recorded isometrically using Grass FT.03 strain gauges and a Grass 7D polygraph. Control preparations were suspended under similar conditions but with the omission of the inhibitor. Contractions were expressed in terms of the initial maximum response.

It was found that significant sensitization to arachidonic acid sometimes occurred such that a very small response might be enhanced several fold on subsequent retesting with the same dose. This phenomenon occurred in about 10% of the tissues assayed. Since it was always associated with a weak initial contraction, data from tissues which developed a maximum response to arachidonic acid of less than 1 g have not been included in the data presented.

Electron microscopy of vessels - detection of endothelium. Segments of canine basilar or middle cerebral artery were prepared as described and fixed overnight in 2% buffered glutaraldehyde and post-fixed in 1% osmium tetroxide buffered to pH 7.4 with Millonig buffer. The samples were dehydrated through graded ethanol solutions and were then transferred, in absolute ethanol, to a Seevac carbon dioxide critical point dryer. The dried tissues were mounted

on aluminum stubs, sputter coated with gold (Edwards Model S150B Sputter Coater), and examined in a Philips 505 scanning electron microscope at 20 kV.

Incubation procedures and assay of eicosanoids by radioimmunoassay. Canine basilar arteries, with endothelium preserved or removed, were dissected free of surrounding tissue, cut into segments 1-2 mm long and the segments were placed in plastic tubes containing 1 ml of Hanks balanced salt solution containing 20 mM HEPES (pH 7.4). Arachidonic acid (3.3×10^{-5} M) was added and the samples were incubated at 37°C for 5 min in a shaking water bath. The tubes were then centrifuged for 30 s at 8000 X g and placed in an ice bath. Portions of the supernatant were removed and frozen at -20°C for subsequent radioimmunoassay. Prostacyclin and thromboxane were measured as their stable hydrolysis products, 6-keto prostaglandin $F_{1\alpha}$ or thromboxane B_2 respectively. Levels of leukotrienes were also estimated. Radioimmunoassay of these compounds was carried out using standard kits (New England Nuclear). The assay for leukotriene C_4 uses an antibody which is reported to cross-react to about 60% with 11-trans-LTD_4, about 55% with LTD_4 but does not cross react significantly with LTB_4. The detection limits of the assays are reported to be 10 pg for 6-keto-prostaglandin $F_{1\alpha}$, 5 pg for thromboxane B_2 and 25 pg for leukotrienes.

Incubation procedures, extraction and assay of leukotrienes by high performance liquid chromatography (HPLC).

Segments of basilar artery prepared as described in the above section were placed in Ca^{2+} and Mg^{2+}-free saline and then pre-incubated for

20 min at 37°C in Hanks balanced salt solution containing 20 mM HEPES. The tissue segments were then washed and incubated with arachidonic acid (3.3×10^{-6} M), indomethacin (10^{-5} M) and the calcium ionophore A23187 (10^{-5} M) for 30 min at 37°C in a final volume of 2 ml. The supernatant was removed, acidified to pH 3.5 with dilute hydrochloric acid and added to a pre-washed Sep-Pak C18 extraction column (Waters Assoc.). The column was washed successively with water, 10:90 (vol/vol) water methanol and finally with 5 ml of methanol alone. This final fraction was collected and the methanol was evaporated in a stream of nitrogen at 40°C. The residue was redissolved in 200 µl of methanol and analysed by HPLC using a Radial-Pak C18 column (Waters Assoc.) pre-treated with disodium EDTA. The samples were eluted at a flow rate of 1 ml/min with methanol/water/acetic acid (68:32:1 vol/vol) at an apparent pH of 4.9. Eluants were analysed at 280 nm. Synthetic leukotrienes B_4, C_4 and D_4 were used as standards. Fractions corresponding to the retention times of authentic LTC_4 and LTD_4 were collected, rechromatographed and subjected to radioimmunoassay as described.

Drugs and chemicals. Arachidonic acid (99% free acid), 5-hydroxytryptamine, nordihydroguaiaretic acid (NDGA), indomethacin and acetylsalicylic acid were all obtained from Sigma. Other drugs employed were ionophore A23187 (Calbiochem), acetylcholine bromide (Eastman-Kodak), FPL 55712 donated by Fisons Pharmaceuticals, BW 755c donated by Wellcome Laboratories and leukotrienes B_4, C_4 and D_4 donated by Merck-Frosst.

Arachidonic acid and all inhibitors were dissolved in ethanol and subsequently diluted. The final concentration of ethanol never exceeded 0.2%.

Data processing. Statistical analysis was performed by analysis of variance using Duncan's new multiple range test for individual comparisons. Slopes and ED50 values were obtained using a procedure developed in our laboratory for analysis of dose-response data (Cook and Bielkiewicz, 1985). The significance level used was $P<0.05$

Results

Responses of basilar and middle cerebral artery to arachadonic acid. The responses of rings of canine basilar and middle cerebral arteries are shown in fig. 1. Middle cerebral arteries develop less tension than basilar arteries and have a higher ED50. In some tissues an initial relaxation to arachidonic acid is observed at low doses, while contraction is always observed at higher doses.

Effects of removal of endothelium. Dose response curves for arachidonic acid in canine basilar artery were obtained for arteries in which careful attempts had been made to preserve the endothelium and arteries where it had been mechanically removed. No differences were observed between these preparations in responses to arachidonic acid The presence of endothelium can usually be demonstrated by the existence of an acetylcholine-induced relaxation (Furchgott and Zawadzki, 1980), however this has proved to be difficult in canine basilar artery. The relaxant response to acetylcholine was small and variable even in tissues where the endothelium had been preserved

The presence or absence of endothelium was thus confirmed by scanning electron microscopy. In tissues where the endothelium has been removed it was clear that no endothelial cells remained in the preparations. However, we have found that preservation of endothelium in small arteries is difficult and that during insertion of the stainless steel wires various amounts of endothelial damage occurs. While the contractions to arachidonic acid do not seem to depend on the presence of an intact endothelium, interpretation of responses to other agents and antagonists could be complicated by this variable. Thus in subsequent studies of contractility the endothelium was always removed.

Effects of inhibitors of cyclooxygenase and lipoxygenase. In order to elucidate the mechanism of the contractions produced by arachidonic acid, we have used acetylsalicylic acid (ASA) and indomethacin as inhibitors of cyclooxygenase (Vane, 1971) and nordihydroguaiaretic acid (NDGA) as an inhibitor of lipoxygenase (Tappel et al. 1953). We have also used BW 755c which inhibits both enzymes (Higgs et al., 1979) and FPL 55712 which is a selective antagonist of SRS-A receptors (Augstein et al., 1973). In control experiments responses to 5-hydroxytryptamine (5-HT) were measured before and after addition of the inhibitors. 5-HT produces contractions by interaction with its own receptors and thus served to confirm the selectivity of the drugs.

Indomethacin at concentrations from 10^{-6} to 10^{-4} M and ASA (1.7×10^{-5} M or 1.7×10^{-4} M) were without effect on the arachidonic acid-induced contractions (fig. 2A and 2B).

NDGA was tested at three concentrations. It lacked any significant effects at 10^{-6} M, while at 10^{-5} M a significant inhibition of the arachidonic acid response was observed. This was even more marked at 10^{-4} M, but this result has a non-specific component in that this concentration of NDGA also inhibited the response to 5-HT. Results with NDGA are shown in fig. 2D.

The compound BW 755c was found to inhibit contractions to arachidonic acid at 10^{-5} M (fig. 2E). A tenfold higher concentration also reduced the response to 5-HT. Finally the compound FPL 55712 which inhibits leukotriene receptors reduced the response to arachidonic acid at doses of 2×10^{-6} M and 2×10^{-5} M without effect on the response to 5-HT (fig. 2C).

Responses of basilar artery to leukotrienes. Dose-response curves to leukotrienes C_4 and D_4 are shown in fig. 3. Cerebral arteries are sensitive to these agents at rather low concentrations, although the response is not large in terms of tension developed. Insufficient material was available to examine the effects of high concentrations of leukotrienes.

Radioimmunoassay of eicosanoids in basilar artery stimulated with arachidonic acid. The following experiments were designed to reproduce the conditions of the contractility studies. Preparations of chopped basilar artery, in amounts roughly equivalent to one ring, were made in which the endothelium has been preserved or mechanically removed. The preparations were incubated with arachidonic acid at the concentration which provided the maximal response in the contractility studies. The supernatants of the

incubations were analyzed by radioimmunoassay for leukotrienes C_4/D_4 and for thromboxane B_2 and 6-keto-$PGF_{1\alpha}$.

The results of these studies are shown in table 1. There is no detectable basal production of leukotrienes in this preparation and thromboxane A_2 is only generated in unstimulated preparations when the endothelium is intact. There is a significant release of prostacyclin, most of which comes from the endothelium, as seen when the incubations are performed with exogenous arachidonic acid. Both thromboxane A_2 and prostacyclin seem to be generated mostly by the endothelium. The fact that the amount of thromboxane B_2 is increased after addition of arachidonic acid to vessels without endothelium suggests that thromboxane can be formed by components of the vessel wall other than the endothelial cells. Arachidonic acid caused a small non-specific cross-reactivity in the leukotriene radioimmunoassay, thus levels of leukotrienes determined in the presence of arachidonic acid were corrected for this interference.

Analysis of leukotrienes by high performance liquid chromatography (HPLC). Studies with inhibitors of the arachidonic acid cascade and with a leukotriene antagonist suggested that these substances may participate in the arachidonic acid-induced contraction of cerebral arteries. To determine which leukotrienes can be synthesized by cerebral arteries, tissues were stimulated with A23187(10^{-5} M), a calcium ionophore, in the presence of arachidonic acid (3.3×10^{-6} M) and indomethacin (10^{-5} M) for 30 min at 37°C. The supernatant from these incubations was extracted and analyzed by HPLC. The results indicated the presence of a peak co-chromato-

graphing with LTC_4 and a small shoulder with the same retention time as LTD_4. No peak corresponding to LTB4 was detected. The HPLC fractions with retention times corresponding to LTC_4 and LTD_4 were further analyzed by radioimmunoassay sensitive to the peptidoleukotrienes. Both fractions from the HPLC eluants showed reactivity in this assay. The amount of LTC_4 released was in the range of 11-18 pg/mg tissue, based on 5 separate experiments.

Discussion

Release of free arachidonic acid from membrane phospholipids follows many types of tissue injury or cell stimulation. Free arachidonic acid is rapidly oxidized by cyclo- or lipoxygenases or non-enzymatically by free radicals generated by events such as hemorrhage or clotting, to products which are intensely vasoactive. This is of particular interest in the case of tissue injury which follows subarachnoid hemorrhage where a prolonged vasoconstriction often arises which is associated with ischemic neurological deficit. While the role of prostaglandins in this system has been examined quite extensively, the role of the lipoxygenase products is not well understood. The addition of exogenous arachidonic acid to isolated blood vessels and pharmacological studies of the response has enabled us to examine the role of arachidonic acid metabolism in the sustained contractions we observe in this preparation.

While there are quantitative differences in the response of basilar or middle cerebral artery to arachidonic acid it seems clear that cerebral arteries are qualitatively similar, while responses in peripheral arteries are mediated by a different mechanism. This is obvious first

in the question of the role of the endothelium which is not essential for contractile activity in cerebral arteries. Very recently in a brief communication Fujiwara et al. obtained a different result. These workers used spiral strips of cerebral arteries, and reported that rubbing of the internal surface abolished the response to 10^{-5} M arachidonic acid. It is surprising that the preparation of spiral strips of cerebral artery could be achieved with preservation of the endothelium, since Furchgott (1983) has shown that even in very large arteries extensive damage results from this procedure. We have measured the tonic response to arachidonic acid which is large and well maintained. The response recorded by Fujiwara was transient and may represent a different phenomenon. In our experiments it is clear that in tissues where there is no trace of endothelium the arachidonic acid response is well maintained.

In agreement with Koide et al. (1981), we find that the response is not significantly inhibited by indomethacin. The response is also resistant to blockade with ASA and this implies that cyclooxygenase products are not responsible for the response to arachidonic acid. The sensitivity of the response to the lipoxygenase inhibitor NDGA and the inhibitor of both pathways BW 755c implies that leukotrienes may be involved, and this is further demonstrated by the sensitivity of the response to FPL 55712 which blocks leukotriene receptors. Koide et al. (1981) showed that the hydroxy- and hydroperoxy-eicosatetraenoic acids produced large and long-lasting contractions of cerebral vessels, and it is possible that the component which is resistant to FPL 55712 arises from the action of these products. Although in preliminary

studies we failed to demonstrate a significant effect of FPL 55712 (Jancar et al., 1986), as the number of observations was increased there was clearly an attentuation of the arachidonic acid response after treatment with this agent. FPL 55712 has been shown to inhibit the constriction of cerebral arterioles to LTC_4 (Rosenblum, 1985). Thus on the basis of contractility studies it would appear that in arteries devoid of endothelium, arachidonic acid produces its contractions by providing substrate for the lipoxygenase pathway, with some but not all the response arising from the action of leukotrienes.

When the release of eicosanoids is studied, the results are consistent with this explanation. While there is little basal release of any compound except prostacyclin, stimulation with arachidonic acid results in an endothelium-independent enhancement of the release of leukotrienes. The cellular origin of arachidonic acid metabolites in the cerebral arterial wall is not clear although in peripheral vessels it does seem that the endothelium is the major source of prostacyclin. Peripheral endothelial cells grown in vitro do show the capability of releasing thromboxane after stimulation, although this is a minor component compared to release of prostacyclin. Our results suggest that the endothelium of basilar artery may contribute to a basal release of thromboxane and that after arachidonic acid stimulation there is an additional release of about 3 pg/mg. In arteries without endothelium, arachidonic acid induces formation of equivalent amounts of thromboxane which suggests that it is released from components of the blood vessel wall other than the endothelial cells. One might suppose that in vessels with intact endothelium the response to arachidonic acid

results from effects of thromboxane A_2 and leukotrienes to give contraction, which is somewhat offset by the relaxant action of prostacyclin. High performance liquid chromatography shows that the major leukotriene produced is LTC_4 with some LTD_4, but no detectable LTB_4. We have estimated that each mg of basilar artery produces approximately 2×10^{-11} M of LTC_4/D_4 upon addition of arachidonic acid. Considering that the basilar artery starts responding to 5×10^{-11} M LTC_4 or LTD_4 the amount released by one ring of artery should be sufficient to cause significant effect on these vessels. The intermediate HPETEs and HETEs may also play a role in the generation of the response. It was reported that 15-HPETE possesses strong vasoconstrictor activity in cerebral arteries both in vitro (Koide et al., 1981) and in vivo (Sasaki et al., 1981). Also, during platelet aggregation, which may occur after cerebral hemorrhage, a large amount of 12-HETE is expected to be formed which may also contribute to the contractile response of cerebral arteries.

These data may be relevant to the pathophysiology of cerebrovascular spasm following subarachnoid hemorrhage, where endothelial damage is evident and release of arachidonic acid from platelets and other cells such as erythrocytes of the clot (Baker and Loh, 1987) may occur. Damage to the endothelial lining will result in a decrease in the formation of the vasodilator prostacyclin, whereas arachidonic acid may be converted by the cerebrovascular wall to contractile lipoxygenase products, including the leukotrienes.

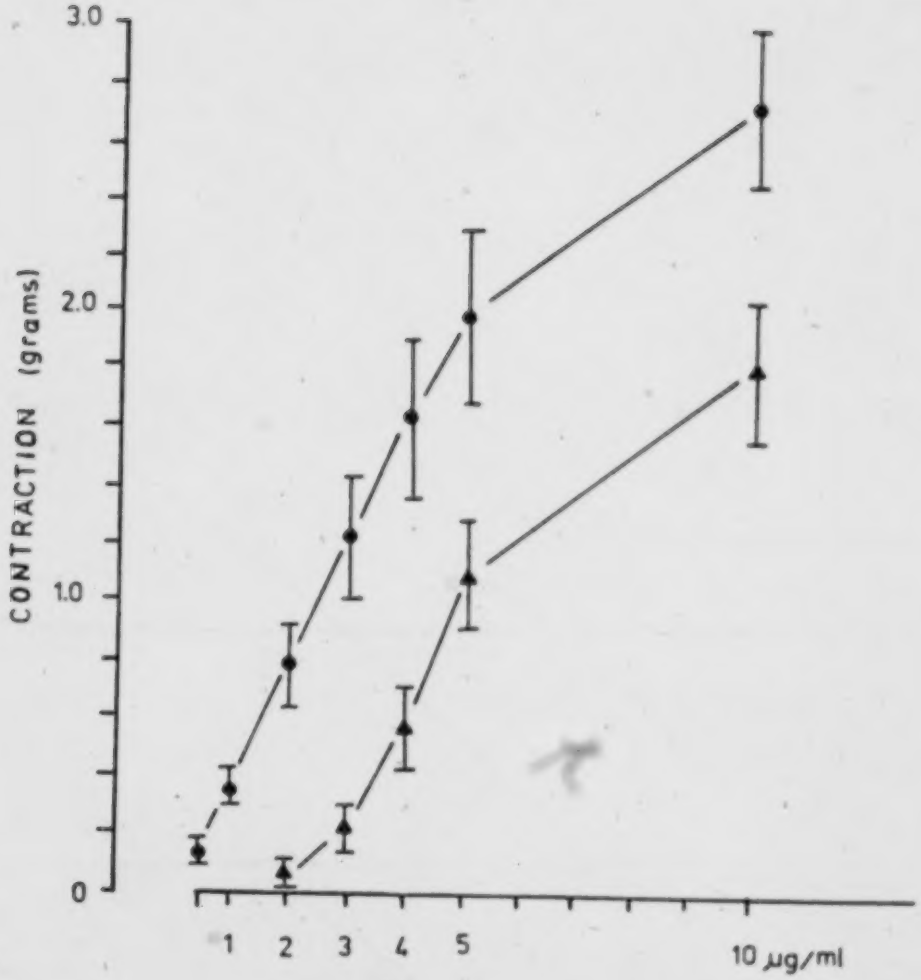


Fig. 1 Concentration-response curves for arachidonic acid in canine basilar (●) and middle cerebral (▲) arteries. Developed tension (grams) as ordinate, concentration of arachidonic acid as abscissa. Bars represent S.E. (N≥12)

Fig. 2 Cumulative concentration-response curves to arachidonic acid. Percentage initial maximum response as ordinate, concentration of arachidonic acid as abscissa. Control values in the absence of antagonist always shown as closed circles (●). For A,B, and C mean absolute value ± SE corresponding to 100% tension development is 1.77 ± 0.25g (N=12); for D and E it is 2.73 ± 0.28g (N=12).

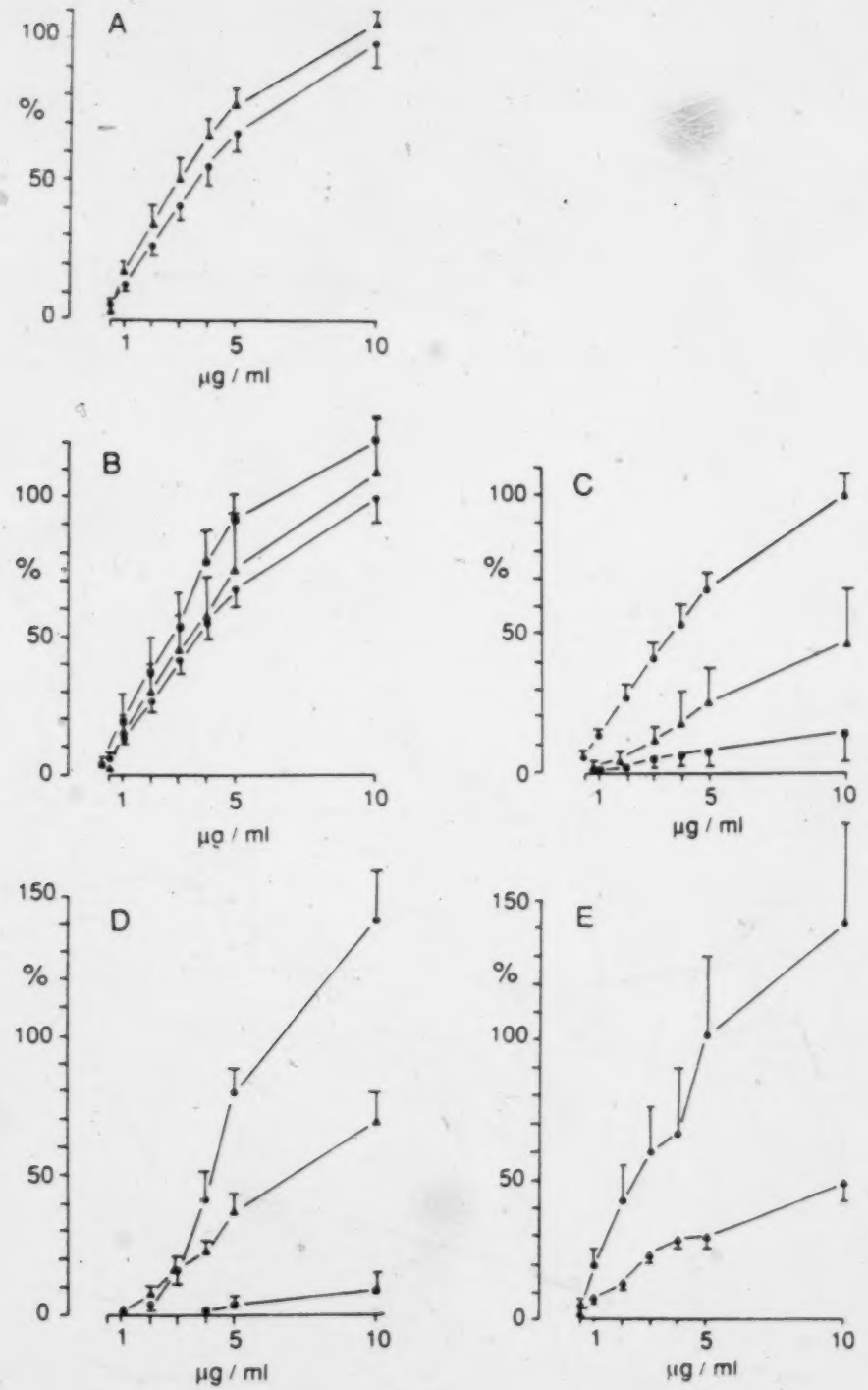
A: after 10^{-5} M indomethacin (▲).

B: after 1.7 X 10^{-5} M (▲) or 1.7 X 10^{-4} M (■) acetylsalicylic acid.

C: after 2 X 10^{-6} M (▲) or 10^{-5} M (■) FPL 55712.

D: after 10^{-5} M (▲) or 10^{-4} M (■) NDGA.

E: after 10^{-5} M BW 755c (▲). Bars represent standard errors ($N \geq 8$)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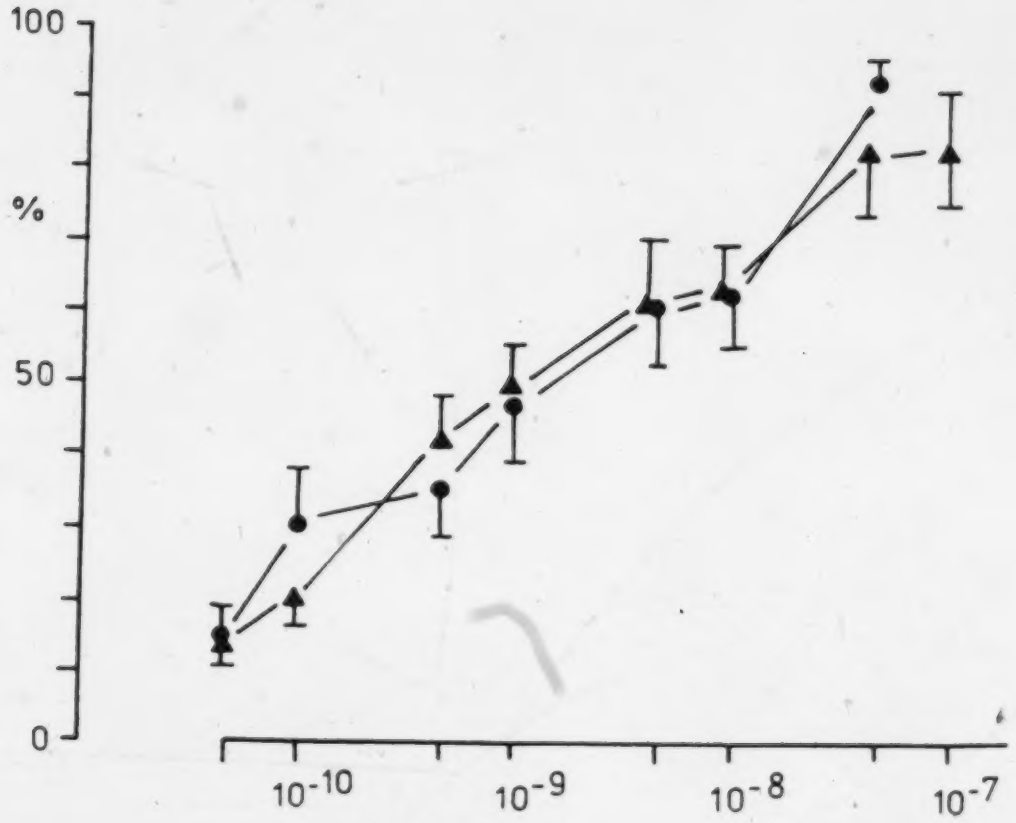

Fig. 3 Cumulative concentration-response curves to leukotriene C_4 (●) and leukotriene D_4 (■) in canine basilar arteries. Percent response relative to 10^{-7} M LTC_4 or LTD_4 (= 100%) as ordinate, molar concentration of leukotriene as abscissa. Bars represent S.E. Mean absolute values ± S.E. corresponding to 100% tension development are 1.87 ± 0.22g (LTD_4, N=9) and 1.71 ± 0.30g (LTD_4, N=10).

TABLE 1 Release of eicosanoids by dog basilar artery stimulated with arachidonic acid as measured by radioimmunoassay†

	picogram/mg of tissue			
	BA (endo$^+$) + AA	BA (endo$^-$) + AA	BA (endo$^+$)	BA (endo$^-$)
LTC_4	4.73 ± 1.3*	6.95 ± 1.62*	0	0
6-keto $PGF_{1\alpha}$	92.33 ± 15.12°	21.31 ± 8.13	63.67 ± 12.86	26.97 ± 15.59
TXB_2	5.77 ± 0.99°*	2.36 ± 0.55*	2.71 ± 0.43°	0

† Basilar artery (BA) with endothelium preserved (endo$^+$) or mechanically removed (endo$^-$) incubated with 3.3 X 10^{-5} M arachidonic acid (AA) for 5 min at 37°C. Results are expressed as mean ± S.E. of 6 tissues from 3 dogs.

* $p < .05$ compared to equivalent preparation without arachidonic acid.

° $p < .05$ compared to equivalent preparation with endothelium removed.

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CHAPTER 5

RELEASE OF LEUKOTRIENES FROM ISOLATED CEREBRAL ARTERY

CHAPTER 5

RELEASE OF LEUKOTRIENES FROM ISOLATED CEREBRAL ARTERY[1]

It has been known for a number of years that arachidonic acid can be transformed into a variety of biologically active products by two separate pathways, one of which, the cyclooxygenase pathway, yields a series of compounds collectively referred to as prostaglandins, while the other lipoxygenase pathway, yields the more recently discovered group of compounds referred to as leukotrienes. The prostaglandins have a wide range of different biological activities, and are known to have dilator or constrictor activities on blood vessels depending on the specific agent and the vascular bed involved. This is of particular interest in the cerebral circulation where an imbalance in the production of vasodilator prostacyclin and vasoconstrictor thromboxane is believed to be responsible for the sustained constriction of cerebral vessels which occurs some days after subarachnoid hemorrhage (1). This condition, which is referred to as cerebral vasospasm, is likely to develop as a result of a complex series of changes in the vessel, possibly triggered by the release of hemoglobin from lysed erythrocytes (2,3). The role of the lipoxygenase pathway products has been less well studied although there are some reports of the activity of exogenously administered

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[1]A version of this chapter has been published. Schulz, R., Jancar, S. and Cook, D.A. (1986) Proc. West. Pharmacol. Soc. 29: 101-104.

preparations of leukotrienes in cerebral artery (4,5). Furthermore, after stimulation with arachidonic acid and the calcium ionophore A23187, rat brain releases leukotrienes C_4, D_4 and E_4 (6) while in gerbil brain, after ischemic insult, subarachnoid hemorrhage or concussion, a peptidoleukotriene-like compound is released (7). In each case, however, these compounds seem to arise chiefly from nervous tissue. Peripheral arteries are known to release a LTD_4-like compound mostly from the adventitia (8) while recently it has been discovered that the vascular endothelium can synthesize the allied compounds 12- and 15-HETE (9).

The leukotrienes have profound effects in a variety of smooth muscle preparations and it thus seemed relevant to ask whether intact cerebral artery has the capability of synthesizing leukotrienes. We have thus used HPLC and radioimmunoassay to assess the production of leukotrienes by incubating rings of canine cerebral arteries with the ionophore A23187 to stimulate production of these compounds. We report here preliminary results of these studies.

Methods

Dogs were anesthetized with pentobarbitol and the brain was removed and the basilar or middle cerebral arteries were cleaned carefully, cut into 1 mm sections and rinsed with buffered saline free of calcium or magnesium. Rings were then pre-incubated for 20 min at 37°C in Hanks-Hepes buffer (20 mM) (HHB) in the absence or in the presence of indomethacin (8 μM). Samples were washed with HHB and incubated with arachidonic acid at concentrations from 3-300 μM, the calcium ionophore A23187 (10 μM) and indomethacin (10 μM). Samples

were incubated for up to 60 min at 37°C in a final volume of 2 ml. The supernatant was removed and brought to pH 3.5 by careful addition of 0.1 M HCl. This solution was cooled then added to a pre-washed Sep-Pak C18 extraction column. The column was washed with water (5 ml) then with 10% aqueous methanol (1 ml). The leukotrienes were then eluted with methanol (5 ml) and the methanol was evaporated at 40°C under a stream of nitrogen. The residue was redissolved in methanol (200 µl) and analyzed by HPLC (10) using a pump with a U6K sample injector. A Radial-pak C18 column which had been treated with disodium EDTA (11) was used in a compression module. An ultraviolet spectrophotometer with a 280 nm filter formed the detection unit and peaks were recorded on a Hewlett-Packard 3390 integrator. The column was eluted at a flow rate of 1 ml/min with methanol/water/acetic acid (68:32:1) at an apparent pH of 4.9. The system was standardized using leukotrienes B_4, C_4 and D_4 (LTB_4, LTC_4, LTD_4) from Merck Frosst kindly provided by Dr. M. Saad. Fractions corresponding to the retention times of LTC_4 and LTD_4 were collected and freeze-dried for subsequent radioimmunoassay. The assay was carried out using a commercially available kit which provides an antibody against LTC_4. This antibody is reported to cross-react 60.5% with 11-trans-LTD_4, 55.3% for LTD_4 itself and to have essentially no cross-reactivity with LTB_4.

Results

The HPLC chromatogram of a control incubation of HHB containing A23187 (10 µM), arachidonic acid (300 µM), indomethacin (10 µM) but no tissue is shown in Fig. 1. Retention times for LTC_4, LTD_4 and

LTB_4 standards are 5.6, 9.8 and 10.4 min respectively. It can be seen that the system can resolve the three leukotrienes clearly, although indomethacin, with a retention time of 5.3 min would nearly co-elute with LTC_4.

A representative chromatogram from a 60 minute incubation of cerebral artery with similar medium to that described for Fig. 1 but with no indomethacin, is shown in Fig. 2. A significant peak is observed at 5.6 min corresponding to LTC_4 and a small shoulder with a retention time of about 9.8 min can also be seen, which could correspond to LTD_4. There is no evidence of production of LTB_4 since there is no significant peak with a retention time corresponding to the standard. The fractions with retention times corresponding to LTC_4 and LTD_4 were collected and freeze dried. Radioimmunoassay of the HPLC eluants showed cross-reactivity with both fractions when the tissue samples were incubated with both arachidonate and ionophore but no reactivity was detected in samples obtained from tissues which had been incubated in the absence of ionophore. In the presence of arachidonate (3-300 μM) and ionophore (10 μM) cerebral artery can generate about 15 pg LTC_4 and LTD_4 per mg wet weight of tissue. No reproducible effects of indomethacin on leukotriene production were obtained in the present series although it is premature to conclude that cyclooxygenase inhibitors are without effect since there is considerable variability between preparations, and more dose-effect data are required.

Conclusions

While it is established that lipoxygenase products can cause contractile effects in cerebral arteries and it is also known that these

arteries can generate prostaglandins, prostacyclin and thromboxanes, it was not known whether cerebral arteries themselves could actually generate leukotrienes. These studies suggest that at least under optimum conditions of long incubation, and relatively high concentrations of arachidonic acid, the precursor for both pathways, the calcium ionophore A23187 can induce the arteries to synthesize LTC_4 and LTD_4. This is implied by our results with HPLC and confirmed by radioimmunoassay of the appropriate fractions. The concentrations produced are likely to be sufficient to cause significant effects on the vessels in vivo and suggests the possibility that these compounds may play a role in the production of cerebrovascular spasm. This suggestion must be tentative; it is not known whether arteries can generate these compounds under the influence of anoxia or hemoglobin, nor is it clear where the agents are liberated from, since it seems likely that the endothelium of these preparations is preserved.

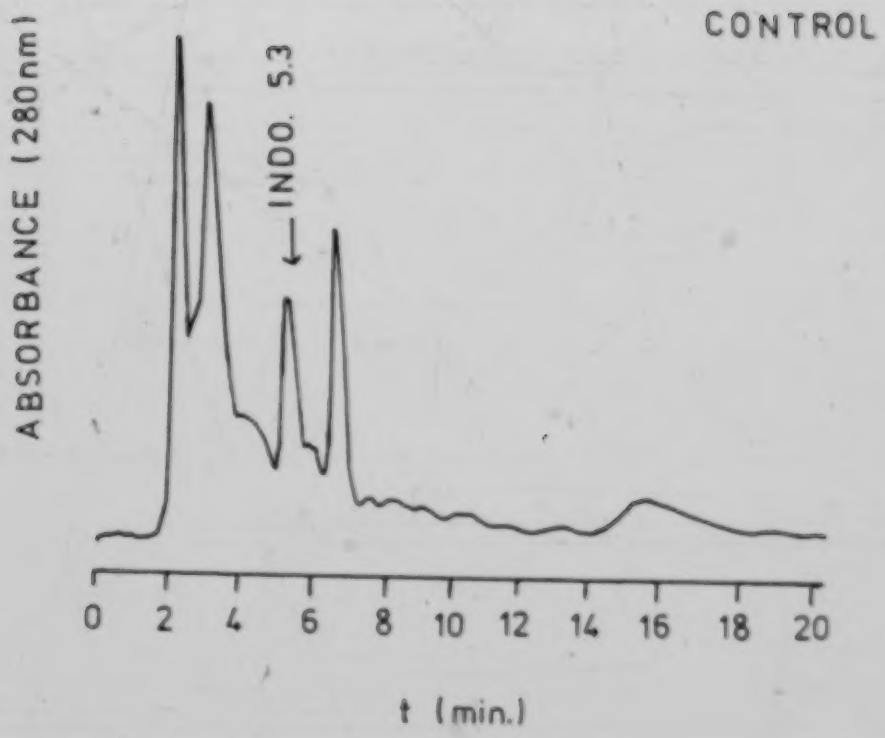

Fig. 1. HPLC chromatogram of control incubation containing buffer, arachidonic acid (300 μM), A23187 (10 μM) and indomethacin (10 μM) but no tissue.

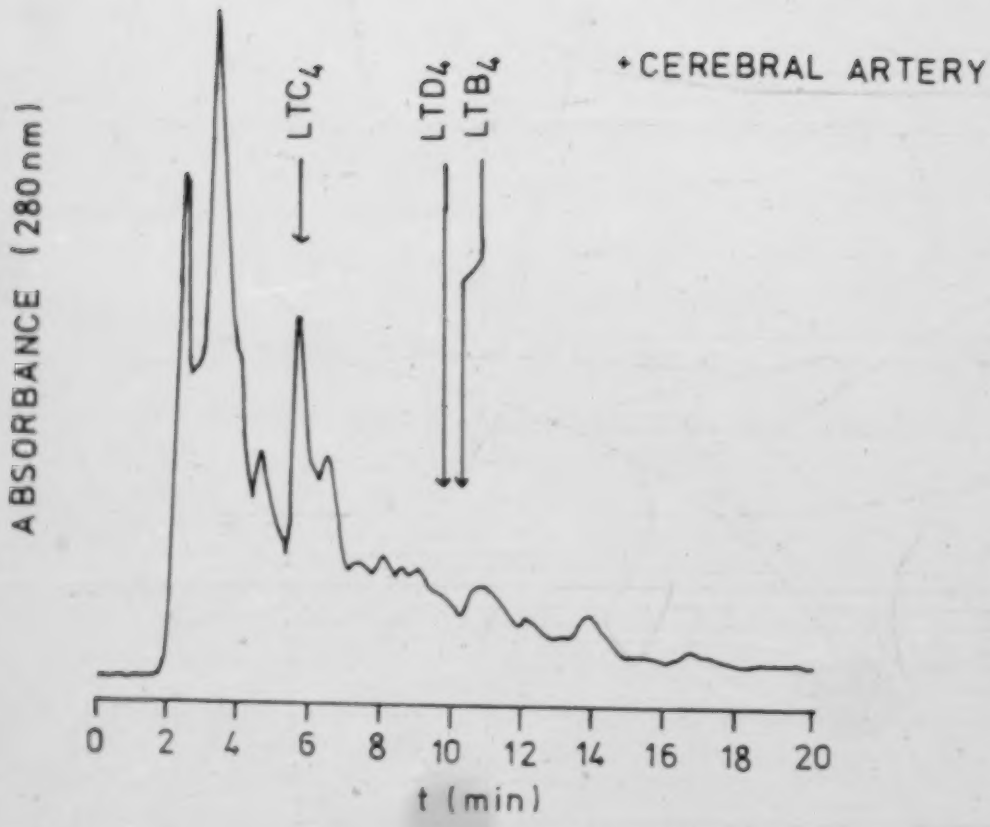

Fig. 2. HPLC chromatogram of products of incubation of rings of canine cerebral artery with buffer, arachidonic acid (300 μM) and A23187 (10 μM) in the absence of indomethacin. Retention times for leukotriene standards are indicated by the arrows. HPLC fractions collected at LTC_4 and LTD_4 elution times correspond to 33 and 9.3 pg/mg wet weight tissue of peptidoleukotriene immunoreactivity, respectively.

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CHAPTER 6

LIPOXYGENASE PRODUCT FORMATION BY CEREBRAL ARTERIES

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LIPOXYGENASE PRODUCT FORMATION BY CEREBRAL ARTERIES[1]

Introduction

Blood vessels can metabolize arachidonic acid to a variety of potent vasoactive products, including the cyclooxygenase-derived prostaglandins as well as the lipoxygenase-derived leukotrienes (for review see Moncada and Higgs, 1986). Both enzyme systems can also lead to simple oxygenated derivatives of arachidonic acid that contain conjugated diene structures, the hydroperoxyeicosatetraenoic acids (HPETEs). HPETEs are quickly reduced to the corresponding hydroxyeicosatetraenoic acids (HETEs) in an aqueous milieu. The HPETEs and HETEs have diverse biological properties, including effects on neutrophil chemotaxis (Goetzl et al., 1977), smooth muscle cell migration (Nakao et al., 1982), inhibition of prostacyclin production (Gryglewski et al., 1976; Setty and Stuart, 1986), stimulation of lipoxygenase activity (Rouzer and Samuelsson, 1986) and direct actions on blood vessel tone, both in the peripheral (Asano et al., 1979) and cerebral vascular beds (Koide et al., 1982).

Cerebral arteries differ in many respects to those from the peripheral circulation, in their anatomy, physiology and pharmacology. This includes a lack of response to sympathetic stimulation, enhanced sensitivity to the effects of serotonin, dilatory responses to

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[1] A version of this chapter has been submitted for publication.

hypercapnia and hypoxia, and their significant contribution to total cerebrovascular resistance (Owman et al., 1978; Toda and Fujita, 1973; Abboud, 1981). Their unique properties come to light in the investigation of disorders such as the delayed cerebral vasospasm which follows hemorrhage into the subarachnoid space. The role of eicosanoids in the pathogenesis of this disorder is unclear. Much attention has been focused on the role of cyclooxygenase-derived products (see Cook, 1984). Less, however, is known about the capability of cerebral artery to synthesize lipoxygenase-derived eicosanoids.

In a previous study we investigated the mechanism by which arachidonic acid induces contraction in isolated rings of canine cerebral artery (Jancar et al., 1987). These contractions were not affected by inhibitors of cyclooxygenase such as indomethacin or acetylsalicyclic acid, but were attenuated by nordihydroguaiaretic acid and BW 755c, both of which are inhibitors of lipoxygenase and with FPL-55712, a leukotriene receptor antagonist, which partially inhibited the contraction. Radioimmunoassay revealed the release of a small quantity of leukotriene C_4 in response to arachidonic acid, and this metabolite is known to contract cerebral artery. In this study we have investigated further the nature of the lipoxygenase metabolites generated by arachidonic acid stimulation of intact segments of bovine cerebral arteries.

Materials and Methods

Release of HETEs from cerebral arteries. The brains of freshly slaughtered cows were removed from the skull and transported to the

laboratory in ice-cold Krebs-bicarbonate buffer (KBB) of the following composition (in mM): NaCl (118), KCl (4.7), $CaCl_2$ (2.5), KH_2PO_4 (1.2), $MgSO_4$ (1.2), $NaHCO_3$ (25) and dextrose (12). The entire circle of Willis and associated arteries were dissected free of surrounding tissue, washed and sliced in cold KBB into rings 1-3 mm wide. Samples containing 20-30 mg tissue were pre-incubated for 5 min at 37°C with agitation in 1 ml KBB gassed with 95% O_2: 5% CO_2. The buffer was replaced with fresh KBB and the samples incubated either in the presence or absence of arachidonic acid (3-330 μM) for 1 to 30 min. Samples containing only buffer and arachidonic acid, or boiled cerebral artery segments and arachidonic acid were used as controls for the non-enzymatic conversion of arachidonic acid to oxygenated by-products. Reactions were ended by centrifugation for 1 minute at 6000 x g. The resultant supernatant was added to 2 ml of acetonitrile. The extraction and analysis by high performance liquid chromatography (HPLC) for lipoxygenase products of arachidonic acid was performed using the method of Eskra et al. (1986). The recovery of arachidonic acid metabolites in the extraction procedure using octadecylsilica extraction columns (Analytichem) (as measured using HPLC) was as follows: 15-HETE, 63%; 5-HETE, 52%; LTB_4, 95%; LTC_4, 56%; LTD_4, 12%. Reverse-phase HPLC analysis was performed using a Waters instrument with a 10 x 0.46 cm column. The column was packed with Hypersil (Shandon) with a particle size of 3 μm obtained from Chromatographic Sciences, Canada. For the purpose of fraction collection, an equivalent but longer column giving greater retention times for all components was used (30 x 0.39 cm; Spherisorb ODS-2;

5 μm particle size; Chromatography Sciences). The column effluent was passed through a dual cell ultraviolet detector and the absorbance was monitored at both 229 nm (for HETEs) and 280 nm (for leukotrienes). The detection limit was 0.5 ng for the HETEs and 2.5 ng for LTC_4.

Verification of HPLC peak identity. In order to verify the identity of the various arachidonic acid metabolites formed by cerebral arteries, the following experiment was performed. Slices of cerebral artery (150 mg) were incubated for 15 minutes at 37°C in 2 ml of KBB to which 330 μM arachidonic acid was added. Samples were processed for HPLC as described. Fractions were collected at one min intervals for the first 16 min followed by 30 sec intervals for the 16-29 min period. The fractions were dried under a stream of nitrogen and reconstituted in phosphate buffered saline (pH 7.1 for 15-HETE, pH 8.5 for 5-HETE). Duplicate radioimmunoassays were then performed on the reconstituted fractions using commercially available kits for both 15-HETE (Amersham) and 5-HETE (Advanced Magnetics, Cambridge, MA). The antiserum to 15-HETE has a cross-reactivity of 2% with 5,15-di-HETE and 0.1% or less to leukotrienes and other mono- and di-HETEs, while the 5-HETE antiserum cross-reacts 7% with 8-HETE and 1% or less with other leukotrienes or mono- and di-HETEs. The detection limit for the 15-HETE assay is 16 pg and 5 pg for the 5-HETE assay.

Incubations in the presence of drugs affecting eicosanoid synthesis. Slices of cerebral artery were prepared as indicated above, and 20-30 mg portions were placed in vials. Slices were either pre-incubated for 10 minutes at 37°C in 0.5 ml of Hanks buffer, pH

7.40 (Gibco) containing 10 mM HEPES (HHB) or boiled for 10 minutes in the same medium. Samples were washed once and a further 0.5 ml HHB was added, which, in some experiments contained either indomethacin (10 μM), or AA-861 (10 μM), an inhibitor of lipoxygenase (Yoshimoto et al., 1982). The samples were incubated for 30 minutes at 37°C. Arachidonic acid (10 μM) was then added to all samples and the incubation was continued for 15 min. The samples were centrifuged at 6000 x g for 1 min and placed on ice, after which 25 μl of 0.85 M ascorbic acid was added to each supernatant fraction. A sample containing 100 μl was taken from each tube for analysis of both 15-HETE and 5-HETE using the specific radioimmunoassays described above.

Drugs and Chemicals. All solvents were HPLC grade and purchased from Fisher Scientific. Arachidonic acid was diluted immediately before each experiment from 10 mg/ml aliquots in ethanol (Cayman Chemical, Ann Arbor, MI). Indomethacin was obtained from Sigma. 5- and 15-HETE standards were purchased from Cayman; 12-HETE and leukotriene standards were donated by Merck-Frosst (Laval, P.Q.) and AA-861 donated by Takeda Chemical Industries (Osaka, Japan).

Results

Arachidonic acid stimulated HETE release from cerebral arteries. Reverse phase HPLC was used to assay the products released during incubation of cerebral arteries with arachidonic acid. The procedure gave consistent profiles as shown in the bottom panel of Fig. 1. At a wavelength of 229 nm, HETEs can be detected; the corresponding

profile for leukotriene detection is at 280 nm and this region showed little evidence of leukotriene production under these experimental conditions. The most abundant products had retention times of 9.5 min and 12.6 min. These corresponded to the authentic standards for 15-HETE and 5-HETE, respectively, as shown in the top panel of Fig. 1 which shows the HPLC profile where 5 ng of each standard had been injected. The detection limit in this assay for each HETE was approximately 0.5 ng. The peak with a wide shoulder having a retention time of 10.7 min corresponds to the retention time of 12-HETE, but was not consistently present and probably represented the presence of a few contaminating platelets in the preparation of cerebral artery. The profile for the control incubation where arachidonic acid was added to the buffer alone is shown in the middle panel of Fig. 1. Small peaks with retention times corresponding to 15-HETE and 5-HETE were observed. These are likely to represent auto-oxidation products of arachidonic acid, and these background values were always subtracted from the total HETE release in subsequent analysis.

Verification of HETE release. Evidence to support the identity of HPLC peaks with the retention time of authentic HETEs as a result of incubation of cerebral artery with arachidonic acid was obtained in one experiment where the HPLC eluent was collected in fractions, and radioimmunoassay for 15-HETE and 5-HETE was performed on each fraction (Fig. 2). The major peaks of immunoreactivity in each case corresponded to the retention times of authentic 15-HETE and 5-HETE. In the case of 15-HETE it appeared that there was some additional

immunoreactive product in the shoulder of the largest peak as seen in the lower panel. This likely represents still unreduced 15-HPETE in this large scale incubation, as this product would have a retention time slightly longer than 15-HETE with reverse phase HPLC. Indeed the antibody to 15-HETE is reported to show 41% cross-reactivity with 15-HPETE in the radioimmunoassay.

Time course of HETE release. In a series of three experiments the time course of HETE release from cerebral artery was determined using 100 μM arachidonic acid to stimulate HETE production (Fig. 3). There was a rapid formation of both products after one minute of incubation and this rose to a plateau level for both products at 15 min with no further subsequent increase. The concentrations of products achieved were: 15-HETE, 353±113 pg/mg tissue; 5-HETE, 223±52 pg/mg after 15 minutes incubation. Further investigation of the dose-response relationship of HETE release and influence of drugs affecting eicosanoid synthesis was therefore carried out using 15 min incubations.

Concentration-response relationship. The release of HETEs as measured by HPLC is shown, as a function of arachidonic acid concentrations between 3 and 330 μM, in Fig. 4. There was no discernable release of either 5- or 15-HETE at a concentration of 3 μM but there was significant release of both metabolites at higher concentrations which rose to a maximum of 1720±541 pg/mg wet weight tissue 15-HETE and 995±275 pg/mg 5-HETE at the maximum dose arachidonic acid (330 μM) which was tested.

Effects of drugs influencing arachidonic acid metabolism. We investigated the enzymatic path of HETE formation in arachidonic acid stimulated cerebral artery segments by measuring the release of both 5- and 15-HETE in the presence of AA-861 (10 μM), an inhibitor of lipoxygenase, or indomethacin (10 μM), a cyclooxygenase inhibitor (Table 1). Specific radioimmunoassays for both HETEs with greater sensitivity of these metabolites than that detected by HPLC were used. This allowed us to reproducibly test the effect of inhibitor drugs using 10 μM arachidonic acid to stimulate eicosanoid formation. Use of the cyclooxygenase inhibitor indomethacin did not significantly alter the release of either HETE from control levels, whereas 15-HETE release was reduced to 10% and 5-HETE reduced to 25% of control in the presence of AA-861. In one experiment where 15-HETE release was measured in duplicate samples, the inhibitor of cytochrome P-450 dependent monooxygenase, SKF525A (33 μM), gave 129% and 94% of control.

Under these experimental conditions the release of non-enzymatically formed HETE, as determined using cerebral artery slices which had been boiled for 10 min was 49±5% and 46±7% of the corresponding levels obtained using unboiled tissue, for 15-HETE and 5-HETE, respectively (N=3).

Similar results were obtained with HPLC for the analysis of HETE release in a separate series of experiments where arachidonic acid at a concentration of 100 μM was used for the stimulus. In three experiments 15-HETE formation was reduced to 56±14% of control values and 5-HETE formation to 44±26% of control values in the presence of

1 μM AA-861. In one experiment acetylsalicyclic acid at 100 μM potentiated 15-HETE release to 380% of control values and 5-HETE release to 181% of the level obtained in the absence of drug.

Discussion

After stimulation with exogenous arachidonic acid, cerebral arteries show a remarkable capacity to synthesize hydroxyeicosatetraenoic acids, including 15-HETE and 5-HETE, the former being the major HETE product. The arterial HETEs were identified by reverse phase HPLC and by specific radioimmunoassays. The formation of 12-HETE, on the other hand, was not consistently observed in these experiments and its appearance may result from platelets which may be present in the artery segments. Platelets are known to generate 12-HETE (Hamberg and Samuelsson, 1974).

The HPETEs and HETEs may play a significant role in vascular disorders such as cerebral vasospasm following subarachnoid hemorrhage. The HPETEs such as 15-HPETE are known for their potent contractile activity in isolated cerebral artery (Koide et al., 1982) and a mixture of arachidonic acid-derived hydroperoxides, when injected into the subarachnoid space of a dog, produced a delayed contraction of cerebral arteries which mimics the cerebral vasospasm which develops after subarachnoid hemorrhage (Sasaki et al., 1981b). The HPETEs and HETEs are known to inhibit prostacyclin production (Gryglewski et al., 1976; Setty et al., 1986), and production of this vasodilator component of vessel tone is reduced in spastic arteries obtained from animal models of cerebral vasospasm (Maeda et al., 1981; Sasaki et al., 1981b; Nosko et al., 1988). The amount of 15-HETE

released by cerebral arteries prompts the question of whether the newly-discovered lipoxins may also play a role in the development of cerebral vasospasm. Both 15-HETE and its precursor HPETE are substrates for the formation of lipoxins (Samuelsson et al., 1987). The lipoxins show a variety of biological actions distinct from other eicosanoids, such as the release of superoxide anion from neutrophils (Serhan et al., 1984) and a long lasting contraction of lung parenchyma as well as dilation of arterioles in vivo (Dahlén et al., 1987).

The production of 15-HETE and 5-HETE by cerebral artery segments does not arise from autooxidation since their production was greatly reduced when the incubation was carried out using tissues which had been boiled. The production of both HETEs is via lipoxygenase enzymes, since the release of both 15-HETE and 5-HETE was inhibited by 90% and 75%, respectively, by incubation in the presence of 10 μM AA-861, an inhibitor of lipoxygenase (Yoshimoto et al., 1982; Schulz & Seeger, 1986). This agent was reported to be a specific inhibitor of 5-lipoxygenase (Yoshimoto et al., 1982) but has not previously been tested for its activity against 15-lipoxygenase. Our results would suggest that it inhibits this enzyme as well. In contrast, indomethacin (10 μM) did not affect the synthesis of either HETE, showing that these agents do not arise from actions of cyclooxygenase. This is in agreement with previous studies of HETE formation from peripheral arteries. Greenwald et al. (1979) showed that the formation of various HETEs upon incubation of rabbit aorta with arachidonic acid, were not affected by indomethacin but were

reduced by ETYA. Human umbilical artery segments were shown to synthesize both 15- and 11-HETE, the former by action of lipoxygenase and the latter by cyclooxygenase activity (Setty et al. 1986). However, Powell (1982) observed that particulate fractions from homogenized fetal calf aorta could produce 15-HETE and 11-HETE from endogenous arachidonic acid; their release was blocked by indomethacin but not by nordihydroguaiaretic acid. Intact blood vessels appear to synthesize 15-HETE via lipoxygenase, whereas the particulate fraction thereof, containing microsomal-associated enzymes like cyclooxygenase has the capacity to make 15-HETE using that enzyme system.

In the range of arachidonic acid concentration studied, HPLC analysis revealed no release of leukotriene from cerebral artery. Our previous studies (Jancar et al., 1987; Schulz et al., 1986) show that using a more sensitive radioimmunological assay canine basilar artery can indeed release leukotriene C_4 in small but significant amounts when incubated with arachidonic acid and can release larger amounts of both leukotriene C_4 and D_4, when incubated in the presence of both the calcium ionophore A23187 and arachidonic acid. Thus, using more vigorous stimulation protocols and sensitive detection methods, it is recognized that cerebral arteries, as well as those from the peripheral circulation (Piper et al., 1984), have the capability to produce peptidoleukotrienes. In a survey of leukotriene generation by various porcine blood vessels stimulated with A23187, Piper et al. (1988), have shown that cerebral arteries can generate five- to tenfold greater quantities of leukotriene D_4-like material than renal, pulmonary or coronary arteries.

Peripheral blood vessels show an endothelium-dependent contraction to arachidonic acid by the formation of cyclooxygenase-derived metabolites (Singer and Peach, 1983; Miller and Vanhoutte, 1985). Cerebral arteries, however, were shown in our laboratory to contract to arachidonic acid, independent of the presence of endothelium. The mechanism of action was suggested to be due to the formation of lipoxygenase products, as the contractions were not inhibited by either acetylsalicyclic acid or indomethacin, but were sensitive to the lipoxygenase inhibitors nordihydroguiatretic acid and BW755c, as well as the leukotriene-antagonist FPL-55712 (Jancar et al., 1987). A very small quantity of peptidoleukotriene release was observed using radioimmunoassay, but leukotrienes may only be responsible for part of the contraction, as FPL-55712 was not able to inhibit the response completely. This present study shows that cerebral arteries have a remarkable ability to generate HETEs from arachidonic acid; their precursor HPETEs are known for their potent contractile actions in both aorta (Asano et al., 1979) and isolated canine basilar artery (Koide et al., 1982). It is suggested that the contractile activity of arachidonic acid in cerebral artery may largely be due to the formation of HPETEs such as 15-HPETE.

The site responsible for the synthesis of the 15-HETE and 5-HETE in the cerebral artery wall likely includes both the endothelium and smooth muscle layers. Although there are conflicting reports on the enzymatic pathway responsible for HETE formation, cultured smooth muscle cells from aorta can make 15-HETE and 11-HETE upon incubation with arachidonic acid (Bailey et al., 1983) and the production of

15-,.12- and 5-HETE has been reported in cells from rabbit aorta (Larrue et al., 1983). We recently have established the culture of smooth muscle cells isolated from bovine cerebral arteries. Their major HETE product in response to stimulation by arachidonic acid is 15-HETE and its release can be inhibited by nordihydroguiaretic acid but not by indomethacin (unpublished observations). Endothelial cells from the peripheral circulation, including umbilical vein (Hopkins et al., 1984), aorta (Kühn et al., 1985) and coronary artery (Revtyak et al., 1988) show the production of 15-HETE as a major metabolite, with 12-, 11- and 5-HETE isomers being formed in lesser quantities in response to incubation with arachidonic acid.

The ability of cerebral arteries to metabolize arachidonic acid via lipoxygenase to products with potent vasoactivity suggests that they must be given particular attention in future investigations of cerebrovascular disorders. Models of cultured cells from cerebral arteries should help delineate the cellular origin of these products and clarify the relationship between smooth muscle and endothelium in modulating their release.

Fig. 1. Upper panel: HPLC profile of an injection of 15-HETE and 5-HETE standards (5 ng each).

Middle panel: HPLC profile of an extract from a 15 min control incubation of arachidonic acid (300 μM) in Krebs-bicarbonate buffer (KBB).

Lower panel: HPLC profile of an extract from a 15 min incubation of arachidonic acid (100 μM) with slices of cerebral artery (42.5 mg/ml) in KBB. Note the enhanced production of material with retention times of 9.5 and 12.6 min, identical to authentic 15-HETE and 5-HETE, respectively.

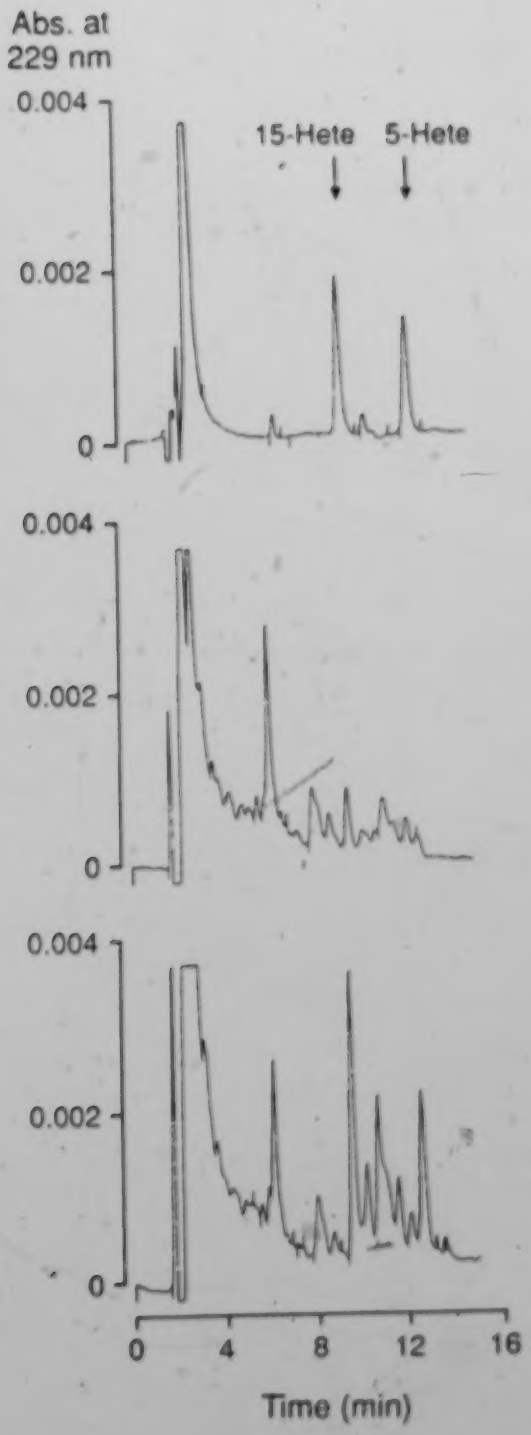
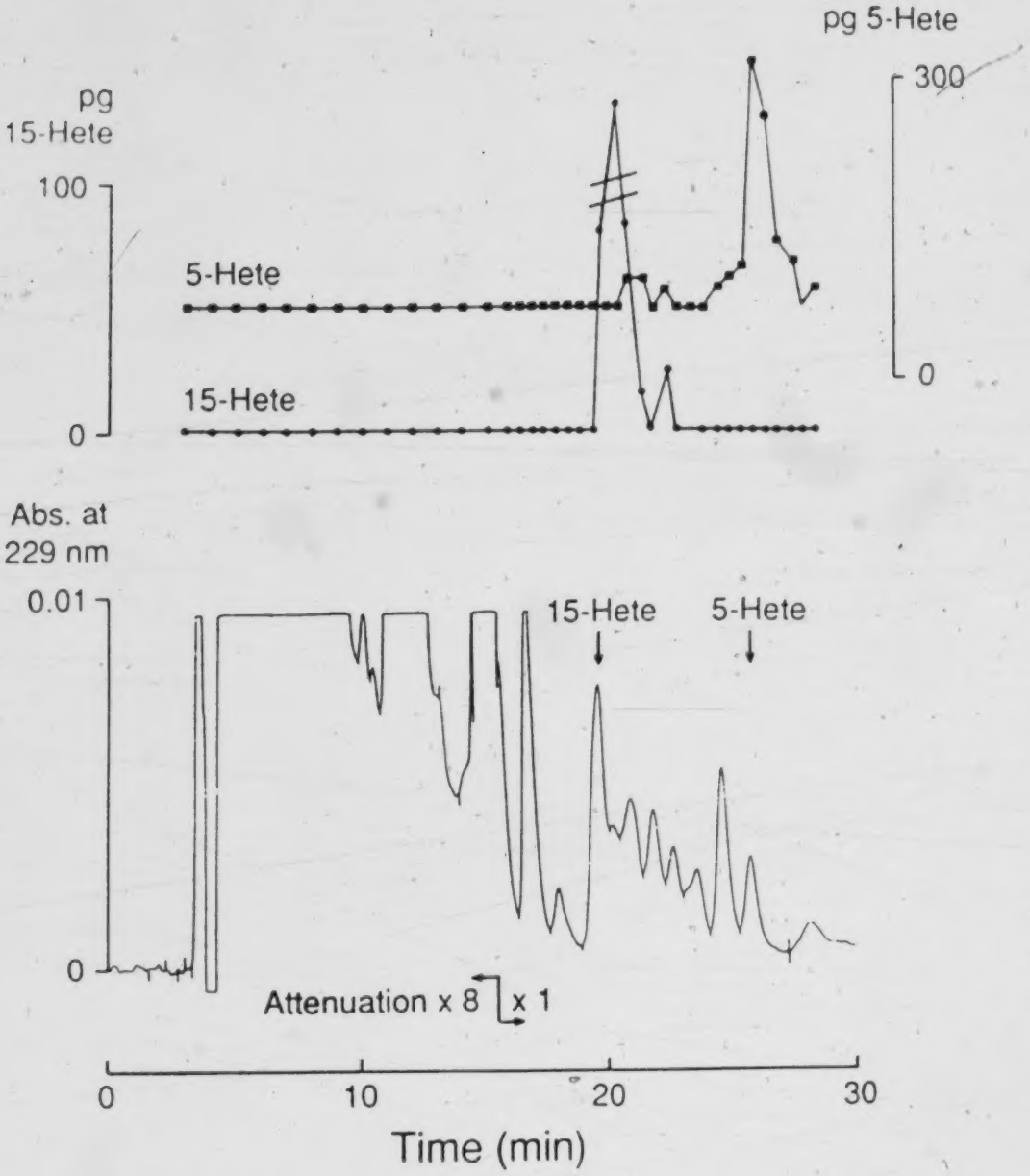

Abs. at
229 nm
0.004
0.002
0
15-Hete
5-Hete
0.004
0.002
0
0.004
0.002
0
0
4
8
12
16
Time (min)

Fig. 2. Lower panel: HPLC profile of an extract from the incubation of cerebral artery slices (82 mg/ml) with 330 μM arachidonic acid for 10 min in KBB. The downward arrows denote the retention times of 15-HETE and 5-HETE standards. The sensitivity of the HPLC integrator was suppressed by a factor of 8 for the first 15.5 min of the chromatogram to show the elution of unidentified polar metabolites.

Upper panel: Immunoreactivity of the HPLC eluent with antibodies to 15-HETE (●) and 5-HETE (■) expressed as pg HETE per fraction. Fractions were collected in one min intervals for the first 16 min followed by 0.5 min intervals for the 16 to 29 min period. The fractions were dried under a stream of nitrogen, reconstituted in phosphate buffered saline and the amount of HETE was determined by specific radio-immunoassays.


pg 5-Hete
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15-Hete
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Attenuation x 8
x 1
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10
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Time (min)

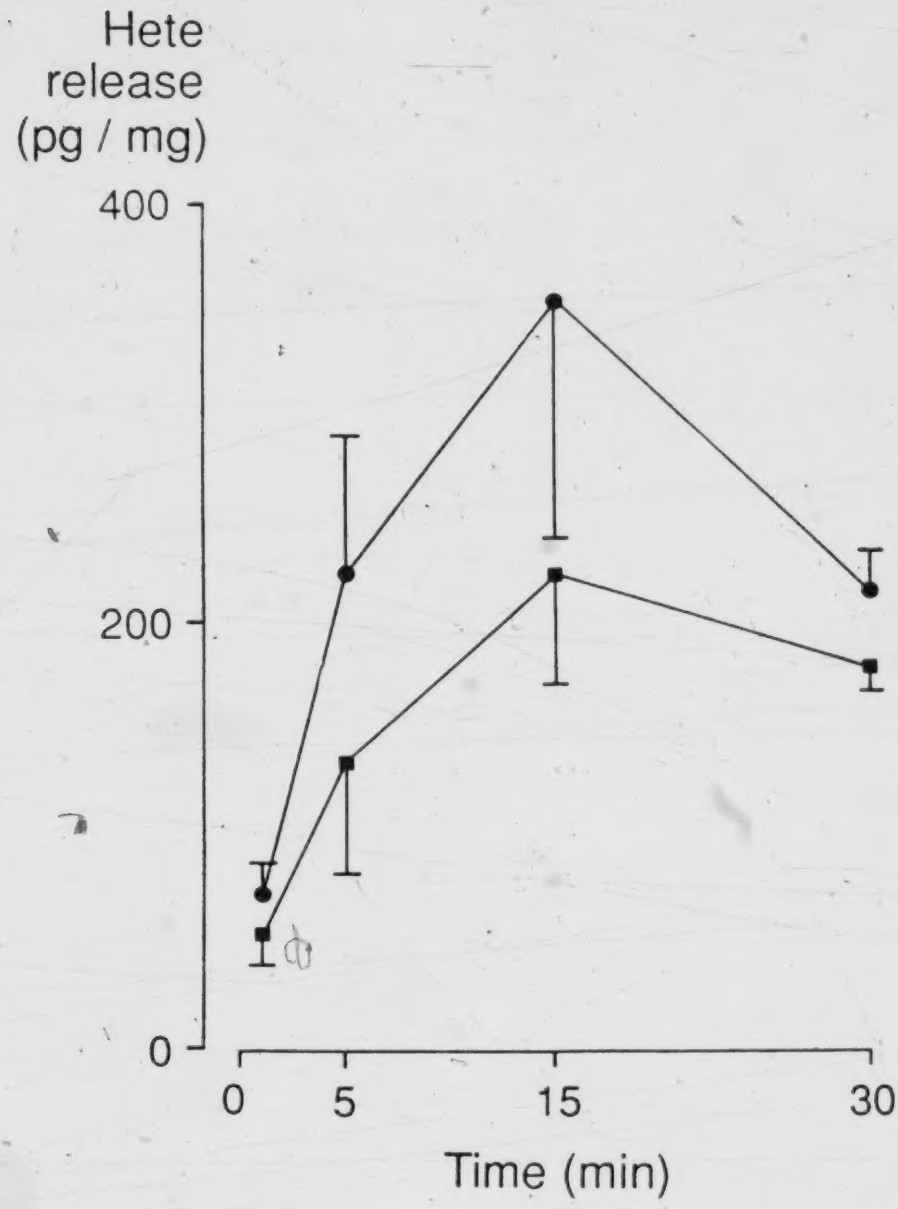

Fig. 3. Time course of HETE release from cerebral artery stimulated with arachidonic acid (100 μM). The amount of HETE released into the incubation buffer, as measured by HPLC, for 15-HETE (●) and 5-HETE (■) is expressed as the average ± S.E. in pg per mg tissue (wet weight) from a total of three experiments.

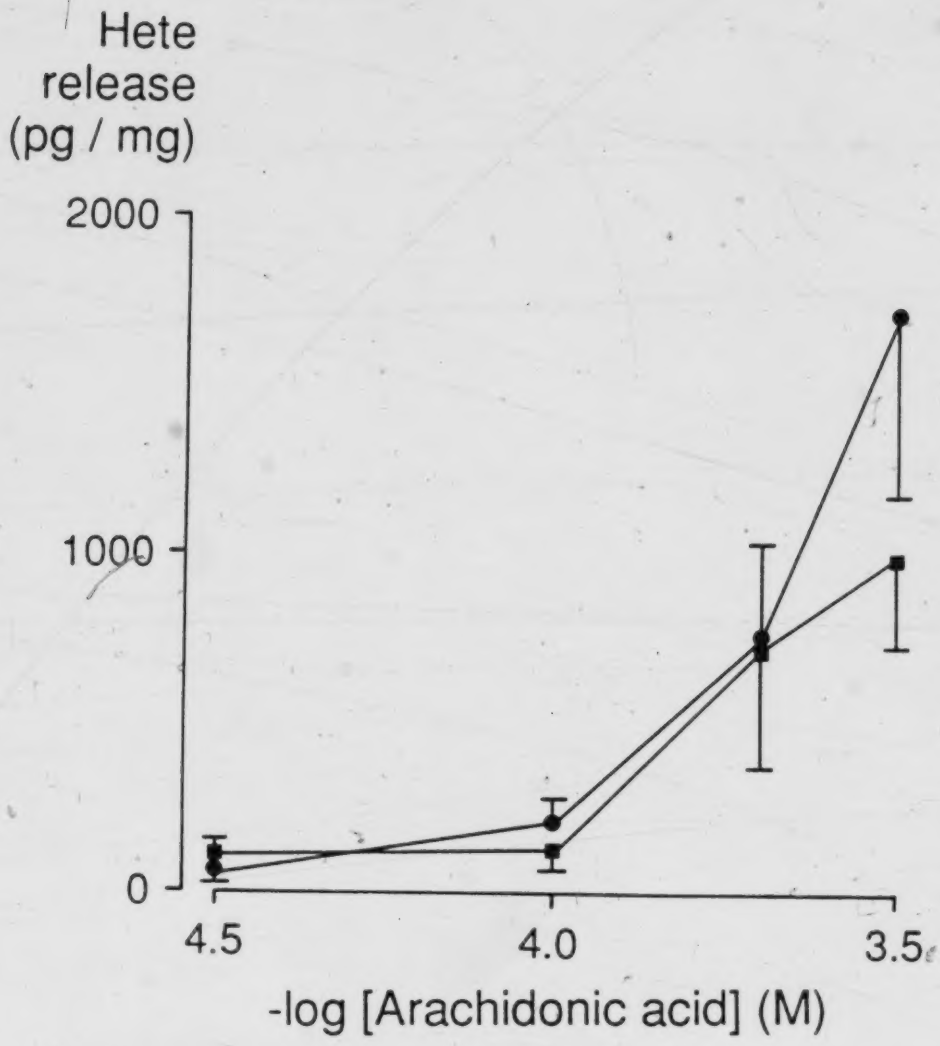

Fig. 4. Concentration-response curves for the release of 15-HETE (●) and 5-HETE (■) from cerebral artery slices incubated with arachidonic acid. The ordinate gives the amount of HETE released as measured by HPLC into the buffer after a 15 min incubation with arachidonic acid, expressed in pg per mg tissue (wet weight) and the abcissa gives the negative logarithm of the molar concentration of arachidonic acid used. Bars denote S.E. from a mean of three to nine experiments.

Table 1 HETE Release by Cerebral Arteries - Effect of Drugs Influencing Arachidonic Acid Metabolism[1]

Drug	15-HETE	5-HETE
Control	34.4 ± 13.6 (4)	16.3 ± 5.0 (3)
Indomethacin, 10 μM	34.5 ± 10.1 (4)	21.6 ± 12.2 (3)
AA-861, 10 μM	3.6 ± 2.8 (4)*	4.1 ± 2.9 (3)*

[1] Data are expressed in pg/mg tissue wet weight ± SE from duplicate determinations in the number of experiments given in parentheses. Cerebral artery slices were incubated 15 min at 37°C with 10 μM arachidonic acid in HHB. Aliquots of buffer were taken for analysis by specific RIA.

* $p<0.05$ compared to control using unpaired t-test.

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CHAPTER 7

SYNTHESIS OF 15-HETE BY CULTURED CEREBRAL ARTERY SMOOTH MUSCLE CELLS

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Introduction

Arachidonic acid is the substrate of two major enzyme pathways leading to a variety of vasoactive products. The cyclooxygenase enzymes are active in the formation of products such as prostacyclin and thromboxane which possess vasodilator and vasoconstrictor properties, respectively. The hypothesis that the vasospasm of cerebral arteries which follows hemorrhage into the subarachnoid space could involve an imbalance between these two arms of the cyclooxygenase pathway as was proposed by Boullin (1) and resulted in a number of studies exploring the formation of prostaglandins by cerebral arteries (2-5), both normal arteries and those that have been exposed to clotted blood in the subarachnoid space. Most authors have observed a reduction in the ability of arteries which were in vasospasm to synthesize the vasodilator prostacylin, which may only be a reflection of the extent of endothelial damage commonly found in spastic arteries, and may not play a causative role in the clinical condition.

Much less is known of the role the lipoxygenase pathway of arachidonic acid metabolism in the events which lead to cerebrovascular spasm. The leukotrienes, lipoxins and hydroperoxyeicosatetraenoic acids (HPETEs) are products of this pathway and have diverse biological activities (see 6,7 for review). The last are unstable in an aqueous enviroment and are quickly reduced to their corresponding

hydroxyeicosatetraenoic acids (HETEs). Cerebral arteries are known to contract in response to leukotrienes (8,9) and also have the capability to synthesize them (9,10). Indeed in a survey of a variety of porcine blood vessels, the cerebral arteries had by far the greatest capacity to release the peptidoleukotrienes after stimulation with the calcium ionophore A23187 (11). A recent report has investigated the presence of leukotriene C_4 in the cerebrospinal fluid from patients with subarachnoid hemorrhage and demonstrated an enhanced level of this vasoconstrictor in the cerebrospinal fluid of patients presenting with vasospasm (12).

The HPETEs and HETEs, first recognized as products from platelets and blood cells, are also formed in the blood vessel wall, as has been shown in blood vessels from the peripheral circulation (13,14). Not only are the HPETEs potent constictors of cerebral arteries in vitro (15,16), they also have been shown produce a condition which mimicks delayed cerebral vasospasm when injected in the subarachnoid space of a dog (17) and are known to inhibit the production of prostacyclin (18). When we investigated the contraction of rings of cerebral arteries to arachidonic acid we found the contraction to be attenuated, at least in part, by inhibitors of lipoxygenase such as nordihydroguaiaretic acid (NDGA), BW 755c and the leukotriene antagonist FPL-55712, but not by inhibitors of cyclooxygenase, such as indomethacin and acetylsalicyclic acid. Analysis of the incubation media of segments of cerebral artery stimulated with arachidonic acid revealed that the arteries release 15-HETE as the major product of the lipoxygenase pathway, as well as some 5-HETE, whereas leukotriene

production was negligible (Chapter 6). Thus the HPETEs may be involved in the contraction of cerebral artery in vitro. Recently, we have developed a technique for the serial cultivation of smooth muscle and endothelial cells from bovine cerebral arteries (Chapter 3). We report here an investigation of the lipoxygenase activity of these cultured cells upon stimulation with arachidonic acid in order to delineate which cell type from the vascular wall contributes to cerebral artery HETE formation.

Materials and Methods

Cell culture. Smooth muscle cells from bovine cerebral arteries were cultured as previously described (see Chapter 3). Briefly, the major arteries of the adult bovine brain were removed, sliced into small segments and slit open on their long axis. The exposed lumen was scraped using a scalpel blade and the blade was dipped into a culture dish containing culture medium (M199, 20% fetal bovine serum, 4mM glutamine, 10 μg/ml streptomycin and 10 U/ml penicillin). Smooth muscle cell identity and culture purity were assessed by light microscopic appearance, immunocytochemical staining with anti-smooth muscle α-actin and electrophysiological characterization of voltage-dependent Ca^{2+} and K^{+} channels. Contamination of cultures by endothelial cells was assessed by staining for Factor VIII-related antigen, and Ulex europaeus lectin binding sites. The cell lines utilized in these studies include BCA 16, 17 and 18 from passages 4 to 22 which showed identical properties characteristic of smooth muscle cells and contained less than 5% endothelial cells.

Endothelial cells from bovine aorta were cultured by the method of Ryan (19). They were cultured in the same medium as above, except with a fetal bovine serum concentration of 10%, and stained positively for Factor VIII-related antigen as well as for Ulex europaeus lectin binding sites, but not for smooth muscle α-actin.

High performance liquid chromatography. Cerebrovascular smooth muscle cells and aortic endothelial cells were cultured in 75 cm^2 flasks and, when confluent, were dislodged from the plates with the use of a rubber policeman. The cells were suspended in phosphate-buffered saline (pH 7.4) to which 10 mM HEPES was added (PBS) and a portion was taken for counting and their viability determined using the trypan blue dye exclusion method. Greater than 95% of the cells were found to exclude the dye in all experiments. The cell suspension was centrifuged for 10 minutes at 200 X g and the pellet was resuspended in Hanks' buffer, pH 7.4 (Gibco) containing 10 mM HEPES (HHB). Portions of approximately one million cells/ml were pipetted into test tubes and were either kept at 37 °C or boiled for five minutes. Arachidonic acid (33 - 300 μM) was added to all tubes and incubations were performed for 15 min at 37°C with gentle agitation. The cell suspensions were centrifuged at 6000 X g for 2 min and the supernatants were added to 2 ml of cold acetonitrile. The extraction and analysis by high performance liquid chromatography (HPLC) for the lipoxygenase products of arachidonic acid was performed using the method of Eskra et al. (20). The sample was centrifuged for 10 min at 2000 X g to precipitate protein and the supernatant was added to 7 ml of 1 mM HCl. This was then passed through octadecylsilica

extraction columns (Analytichem). The column was washed twice with distilled water followed by 20% acetonitrile in water. Using 70% acetonitrile in water the lipoxygenase products were eluted from the column and dried under a stream of nitrogen. The sample was reconstituted in 66% methanol in water and a portion was analyzed by HPLC using a 10 X 0.46 cm column filled with Hypersil (Shandon) with a particle size of 3 μm. The mobile phase was methanol/water/trifluoroacetic acid/triethylamine (80/20/0.1/0.05) at a flow rate of 1 ml/min. With a dual-cell ultraviolet detector the HETEs could be analyzed at a wavelength of 229 nm and the leukotrienes at 280 nm. The detection limit was 0.5 ng for the HETEs and 2.5 ng for LTC_4. Recoveries of arachidonic acid metabolites using this extraction and HPLC method were as follows: 15-HETE, 63%; 5- HETE, 52%; LTB_4, 95%; LTC_4, 56%; LTD_4, 12%.

Analysis of 15-HETE release from smooth muscle cells by radioimmunoassay. To investigate the quantitative aspects of 15-HETE release, smooth muscle cells from cerebral artery in passages 4 to 20 were cultured in 12 well plates (Costar) until confluency was reached. The culture medium was removed, the cells washed with three changes of HHB, and 500 μl of HHB at 37°C was added to each well. The cells were preincubated 5 to 15 min at 37°C with gentle agitation. For experiments with the inhibitors, the cells were preincubated at least 30 min in the presence of the drugs. The inhibitors and their vehicles which were used included: indomethacin (10 μM; aqueous Na_2CO_3), NDGA (10 μM; ethanol/water,1:1) and SKF525A (33 μM; water). To analyze the dose-response relationship of 15-HETE release, arachidonic

acid at a final concentration of 0.1 to 40 μM was added as a dilute solution in ethanol, whereby the final concentration of ethanol never exceeded 0.6% and the vehicle alone was found not to cause any 15-HETE release. For both the time course and enzyme inhibition studies the concentration of arachidonic acid used was 10 μM. Cells were incubated for 15 min or for the times indicated in the time course study. The reaction was terminated by placing the plate on ice, adding 20 μl of ascorbic acid solution (0.85 M) into each well to prevent autooxidation of the samples during the workup procedure. A 100 μl portion of each sample was taken for analysis of 15-HETE content by radioimmunoassay using a commercially available kit (Amersham). The remaining fluid in the wells was removed and the cells were washed once with HHB. A saturated solution of sodium dodecylsulfate in 0.1 M NaOH (0.5 ml) was added to each well to dissolve the cells for later analysis of protein content by the Lowry method (21).

The antiserum to 15-HETE used in the radioimmunoassay had a cross-reactivity of 41% with 15-HPETE, 2% with 5,15-di-HETE and 0.1% or less to leukotrienes and other mono- and di-HETEs. The detection limit of the assay was 16 pg. The presence of ascorbic acid or any of the inhibitors did not cause any interference with the assay.

In all experiments a corresponding well in each plate which did not contain cells was used to perform the same experiment in order to assess the non-enzymatic conversion of arachidonic acid. The background values of 15-HETE obtained from these control wells were

subtracted from the corresponding values obtained from the experiments carried out in the presence of cells.

Materials. Arachidonic acid, 15-HETE, and 5-HETE standards were purchased from Cayman Chemical (Ann Arbor, MI). Indomethacin and NDGA were obtained from Sigma. Leukotriene and 12-HETE standards were a gift from Merck-Frosst (Laval, Quebec) and SKF525A was provided by Dr. R. Coutts.

Statistical analysis. For the comparison of values obtained from treated versus untreated cells, a paired t-test was used.

Results

HPLC analysis of arachidonic acid metabolites. Incubation of cultured cerebral artery smooth muscle cells for 15 min with arachidonic acid gave the typical reverse phase HPLC chromatogram as shown in Fig. 1. With UV detection at a wavelength of 229 nm HETEs are detected; the corresponding tracing with a setting of 280 nm for leukotriene analysis is not shown. Panel A shows the result of an incubation of 1.01×10^6 cells, which were boiled for 10 min before the addition of arachidonic acid (300 μM). Note the presence of small peaks with the retention times of authentic 15- and 5-HETE standards, which the retention times of 5.1 and 6.6 min, respectively. This represents the non-enzymatic formation of these products, probably via autooxidation of arachidonic acid. The arachidonic acid used in this study was itself free of HETE impurities, as determined by HPLC. The lower panel (Fig 1b), shows the corresponding tracing using viable cells under identical conditions. There is a significant elevation in the peak with the retention time of 15-HETE although no change in

the peak coeluting with 5-HETE. The peak immediately before 5-HETE was not identified. Using this method 12-HETE has an elution time of 5.6 min and it was not observed in this or any of the other incubations. In two out of three experiments the only significant product over the background was material coeluting with 15-HETE whereas in one experiment there was no significant stimulation of HETE release. The level of 15-HETE released depicted in Fig. 1 represents 2.95 ng/10^6 cells, corrected for the level observed in incubations of boiled cells with arachidonic acid. The average release of 15-HETE under these conditions was 2.93 ± 0.86 ng/10^6 cells (N = 4).

Scraping cells into suspension did not cause the release of 15-HETE, which could conceivably occur during mechanical stimulation, as was seen from chromatograms where cell suspensions were incubated without arachidonate. The cell membrane was also not disrupted by this procedure as these cells did not take up trypan blue dye. Similar results were also observed if the experiment was performed using cells which had been removed from their culture dishes with the use of collagenase. Under light microscopical examination no deleterious effects to the cells were observed by incubating them as a monolayer culture with 300 μM arachidonic acid for over 15 minutes.

The HPLC profile was verified for the presence of 15-HETE in a related experiment where segments of bovine cerebral artery were incubated with arachidonic acid and the HPLC eluate was fractionated and further analyzed by specific radioimmunoassay for 15-HETE. Biologically synthesized material with the retention time of the 15-HETE standard showed the highest degree of immunoreactivity in the entire fraction set (see Chapter 6).

Concentration-response relationship. To determine the nature of arachidonic acid-induced 15-HETE release from cerebrovascular smooth muscle cells, a rapid and more sensitive radioimmunoassay for 15-HETE was employed which allowed us to make determinations directly from a single well of intact cells attached to their culture surface. The release of 15-HETE as a function of arachidonate concentration after a 15 min incubation from four separate experiments, expressed in units of pg/μg cell protein, is shown by the circles in Fig. 2. At a concentration of 0.1 μM there was no detectable formation of 15-HETE, whereas there was marked dose-dependent rise in 15-HETE release in the range from 1 μM to 40 μM arachidonic acid tested to a maximum of 19.4 ± 5.2 pg/μg protein. The values represent the net formation of 15-HETE, as each test well, performed in duplicate, had a corresponding set of control wells to which an equal dose of arachidonate was added in the absence of cells. The value of non-enzymatic product measured as 15-HETE in the control incubations was subtracted from the value obtained in the presence of cells.

For comparison the experiment was also performed using a culture of endothelial cells from bovine aorta as shown in Fig. 2. These cells as well as those from umbilical vein, are known for their capability to release 15-HETE as the major lipoxygenase metabolite of arachidonic acid metabolism (22,23). They had a similar dose-effect relationship in arachidonate-induced 15-HETE synthesis as compared to smooth muscle cells from cerebral artery, with a maximum of 14.2 pg/μg protein released.

Time-course of 15-HETE formation. In two experiments the time dependence of 15-HETE synthesis by smooth muscle was examined. Fig. 3 shows that after the addition of 10 μM arachidonic acid there was a significant release of the metabolite which rose to an apparent maximum level after 30 min.

Effects of drugs inhibiting arachidonic acid metabolism. In order to determine which enzymatic pathway is involved in the formation of 15-HETE, cells were exposed to either indomethacin (10 μM), NDGA (10 μM), or SKF525A (33 μM), inhibitors of cyclooxygenase, lipoxygenase and cytochrome P-450 dependent monooxygenase, respectively. Incubations were then performed using arachidonic acid (10 μM) to stimulate the cells for 15 minutes. The results with NDGA and indomethacin are shown in Table 1. Expressed as a percentage of 15-HETE release from incubations performed in the absence of inhibitor, indomethacin caused a small diminution of 15-HETE release by 10% whereas NDGA inhibited the release of 15-HETE by 29%. Using a paired t-test comparing the absolute value of 15-HETE release per well, there was no significant effect of indomethacin, whereas NDGA caused a significant attenuation of 15-HETE release.

The effect of SKF525A could not be examined as it was found that this agent had a cytotoxic effect on the cells under the experimental conditions used. Microscopical observation of the cells at the end of the total 45 min exposure time to the drugs showed that SKF525A had caused the smooth muscle cells to detach from the plate and those that remained attached had thin and elongated cytoplasma. This effect was also independent of the addition of arachidonic acid to the cells. The

incubations performed with indomethacin and NDGA showed no change in morphology and no detachment of cells.

Discussion

This study shows for the first time that smooth muscle cells cultured from cerebral arteries have an active lipoxygenase enzyme system which upon stimulation with arachidonic acid produces 15-HETE as its major product. This metabolite is apparently the only HETE which is formed and is released in nanomolar quantities. This was shown using a HPLC method specific for the detection of these products (20) and consistently appeared as the most abundant HETE product. The production of this metabolite was verified by use of a radioimmunoassay which is specific for this eicosanoid. The release of 15-HETE is via the action of a lipoxygenase enzyme, as its formation is reduced using heat-inactivated cells and its synthesis was attenuated significantly by incubating the cells with the lipoxygenase inhibitor NDGA but not the cyclooxygenase inhibitor, indomethacin.

The HPETEs and their corresponding reduced congeners, the HETEs have been recognized to hold a variety of biological actions. Both 15-HPETE and 15-HETE are known inhibitors of prostacyclin synthesis (14,18), an important vasodilator and inhibitor of platelet aggregation, produced chiefly by the endothelium. Trace amounts of the HPETEs can stimulate lipoxygenase activity (24) which can lead to enhanced formation of the leukotrienes, known for their ability to contract cerebral arteries (8,9). The HPETEs have potent vasoconstrictor action on arteries both from the cerebral (15,16) and the peripheral circulation (25). Furthermore both 15-HPETE and 15-HETE

are substrates for the formation of the recently discovered lipoxins (6), which are known to cause a long-lasting contraction of lung parenchyma and dilation of arterioles in vivo (26) and whose actions on the cerebral vasculature are, as yet, unknown.

It is most interesting to speculate whether 15-HPETE/HETE could play a role in the development of delayed cerebral vasospasm following hemorrhage in the subarachnoid space. When a preparation of arachidonic acid hydroperoxides was injected into the subarachnoid space of a dog, an angiographically distinct contraction of the cerebral arteries was observed. The constriction was biphasic, with the initial phase lasting 10 hours after injection, followed by a delayed arterial narrowing which occurred two to three days later and was accompanied by a marked degenerative change in the endothelium (17). 15-HPETE/HETE could cause vasoconstriction both by a direct action (15) as well as causing damage to the endothelium. The endothelium, by releasing both prostacyclin (27) and endothelium-derived relaxation factor (28) is an important vasodilatory balance in the local regulation of blood vessel tone. Studies of the cyclooxygenase activity of cerebral arteries in experimental models of cerebral vasospasm have shown that arteries which have been in spasm have a reduced capability to synthesize prostacyclin (2-5), which could simply reflect endothelial damage or an action of 15-HPETE/HETE which may be present.

Previous studies investigating arachidonate metabolism in smooth muscle cells from peripheral arteries revealed that 15-HETE is a major product. Bailey et al. (29) showed that after prostacyclin, both

15-HETE and 11-HETE were the most abundant metabolites released after incubation of aortic smooth muscle cells from the rat with arachidonic acid, and that all three products were reduced when the cyclooxygenase inhibitors acetylsalicyclic acid or indomethacin were present. In contrast, homogenized smooth muscle cells from rabbit aorta released predominantly 15-HETE, as well as the 12- and 5-HETE isomers upon incubation with arachidonate, and their release was blocked by NDGA but not by indomethacin (30). Our study suggests that NDGA, but not indomethacin, inhibits the release of 15-HETE implying that it is formed by action of a lipoxygenase. Whether these studies reflect differences in arachidonic acid metabolic pathways between the cerebral and peripheral vasculatures in regards to the enzymatic path of HETE formation is unclear.

It has been suggested that oxygenated arachidonic acid derivatives may be synthesized by blood vessels via cytochrome P-450-dependent monooxygenase activity located primarily in the endothelium (31). This enzyme system has been found in aortic tissue from several species (32). We could not evaluate the effect of SKF525A, which inhibits this enzyme system (33) as this drug had a cytotoxic effect on the cells. We found that this agent did not inhibit the formation of 15-HETE from segments of bovine cerebral artery incubated with arachidonic acid (see Chapter 6).

Using HPLC, no leukotrienes were detected after incubation of the cells with arachidonic acid, although cerebral arteries do have the capability to synthesize the peptidoleukotrienes when stimulated with the calcium ionophore A23187 (10,11). Low levels can also be detected

after stimulation with arachidonate alone if a more sensitive radioimmunoassay is used for their detection using only arachidonate to stimulate (9).

Results using sliced whole cerebral artery showed that upon incubation with arachidonic acid they release mostly 15-HETE as well as 5-HETE as the major products of the lipoxygenase pathway (Chapter 6). The release of both products was diminished in the presence of AA-861, an inhibitor of lipoxygenase (34), but not by indomethacin. The ability of smooth muscle from cerebral artery to synthesize 15-HETE has shown us that these cells are one source of this eicosanoid from the cerebral artery wall. The endothelium from peripheral arteries is recognized to synthesize HETEs with 15-HETE as the major product (22,23). The contribution of endothelium is unknown in regards to HETE release from the major cerebral arteries. Whereas studies using cerebral microvessels show that they release 12-HETE in significant amounts, the cultured endothelium thereof only have a minor capacity to synthesize this product (35), and the cells actively incorporate and metabolize this eicosanoid (36).

It is of interest to speculate whether the production of HPETEs/HETEs by cerebral artery smooth muscle cells could contribute to the development of cerebral vasospasm. These arachidonic acid derivatives have also been postulated to be formed by activated oxygen species which may arise during the degradation of the perivascular blood clot (37). One source of free arachidonic acid may be from the lipid released from the red blood cells of the clot (38). Initating amounts of lipid peroxides formed via free radical mechanisms may

activate the lipoxygenase present in the cerebral artery wall as has been shown for purified 5-lipoxygenase (24) and this may result in further HPETE/HETE as well as leukotriene production, causing vasoconstriction by both direct action on the arteries as well as by inhibiting prostacyclin release (vide supra). The possibility of the metabolism of arachidonic acid to lipoxygenase products as well as free radical generating mechanisms playing a synergistic role in the development of delayed arterial narrowing must certainly be investigated.

The pathogenesis of cerebral vasospasm is unlikely to be the result of a single vasoactive agent. With the availability of cultured cells from the cerebral artery wall we now have a valuable tool to better define the individual steps in its development. This will allow us a better understanding of the clinical picture as well as studies performed using experimental models of cerebral vasospasm.

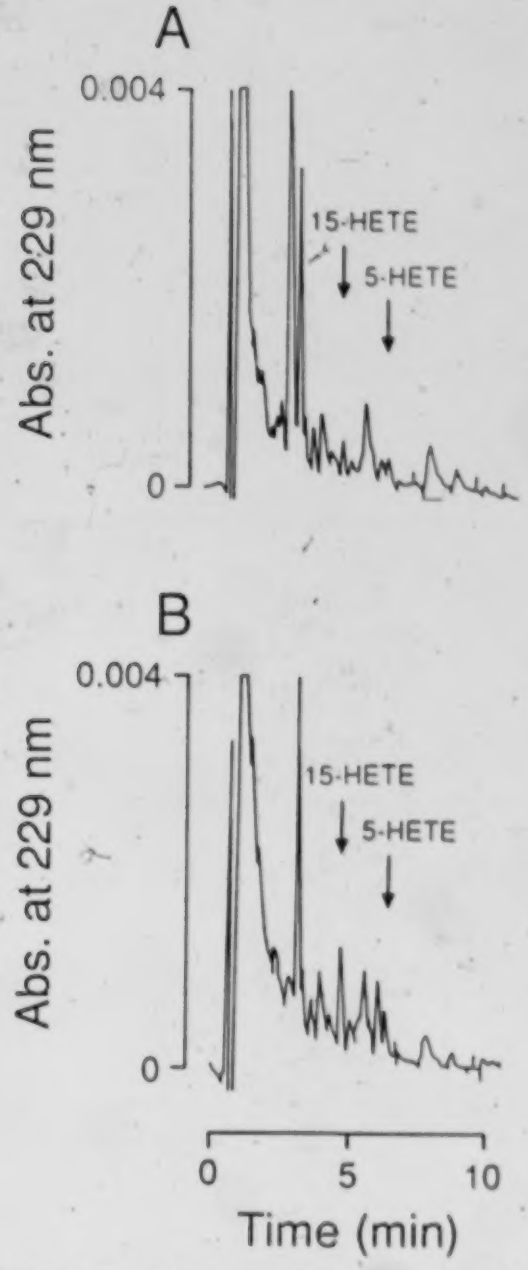
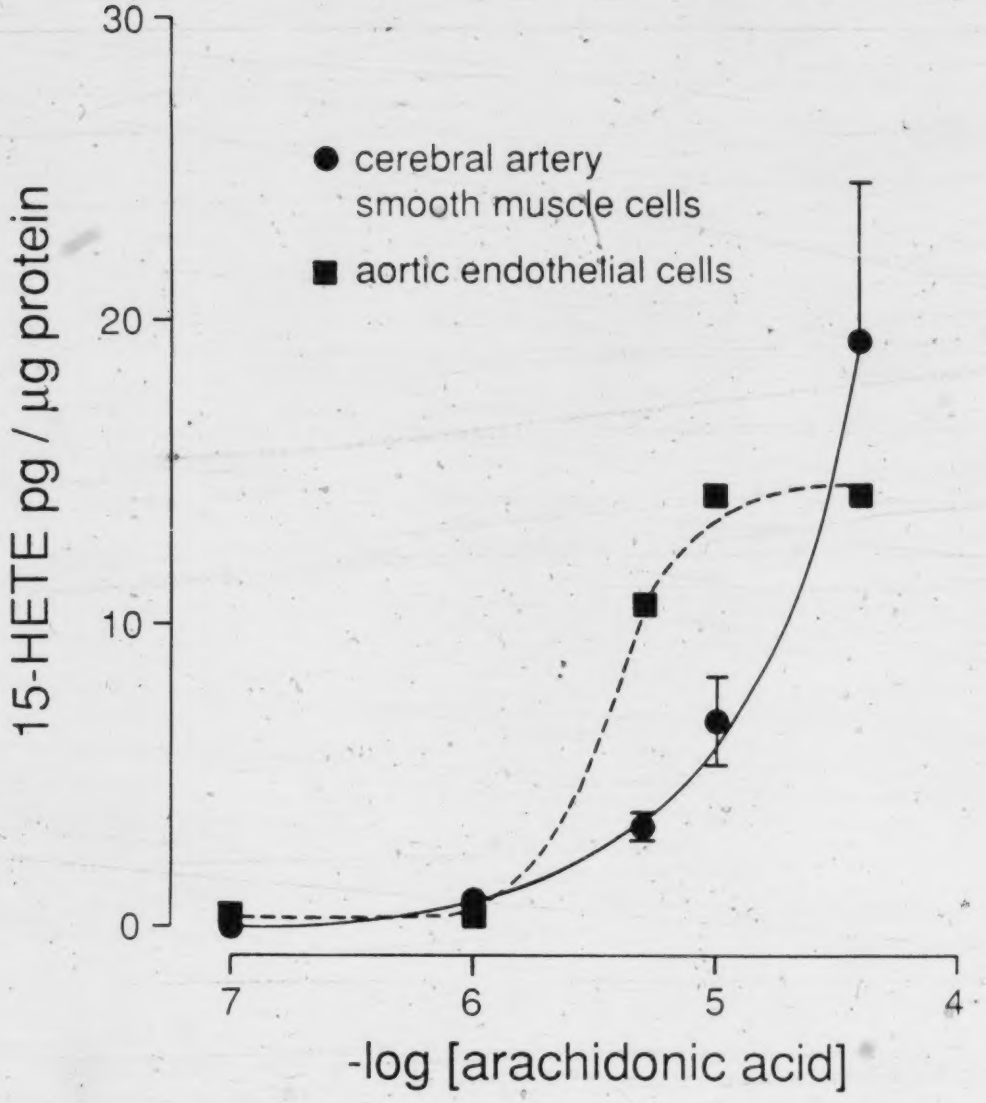


Fig. 1 HPLC tracings from incubations of cerebral artery smooth muscle cells (1×10^6) with arachidonic acid (300 μM). Retention times of HETE standards are shown by the arrows. A) Incubation of cells which had been boiled for 10 min before addition of arachidonic acid
B) Result obtained using viable cells

Fig. 2 Concentration-response relationship of arachidonic acid-induced 15-HETE release from cerebral artery smooth muscle cells and aortic endothelial cells as measured by radio-immunoassay, expressed as pg 15-HETE/μg cell protein

(●) Net 15-HETE release from cerebral artery smooth muscle cells, each point representing the mean ± S.E. of duplicate determinations averaged from four separate experiments

(■) Net 15-HETE release from aortic endothelial cells, each point representing the mean of duplicate determinations in one experiment


30
20
10
0
15-HETE pg / µg protein
cerebral artery
smooth muscle cells
aortic endothelial cells
7
6
5
4
-log [arachidonic acid]

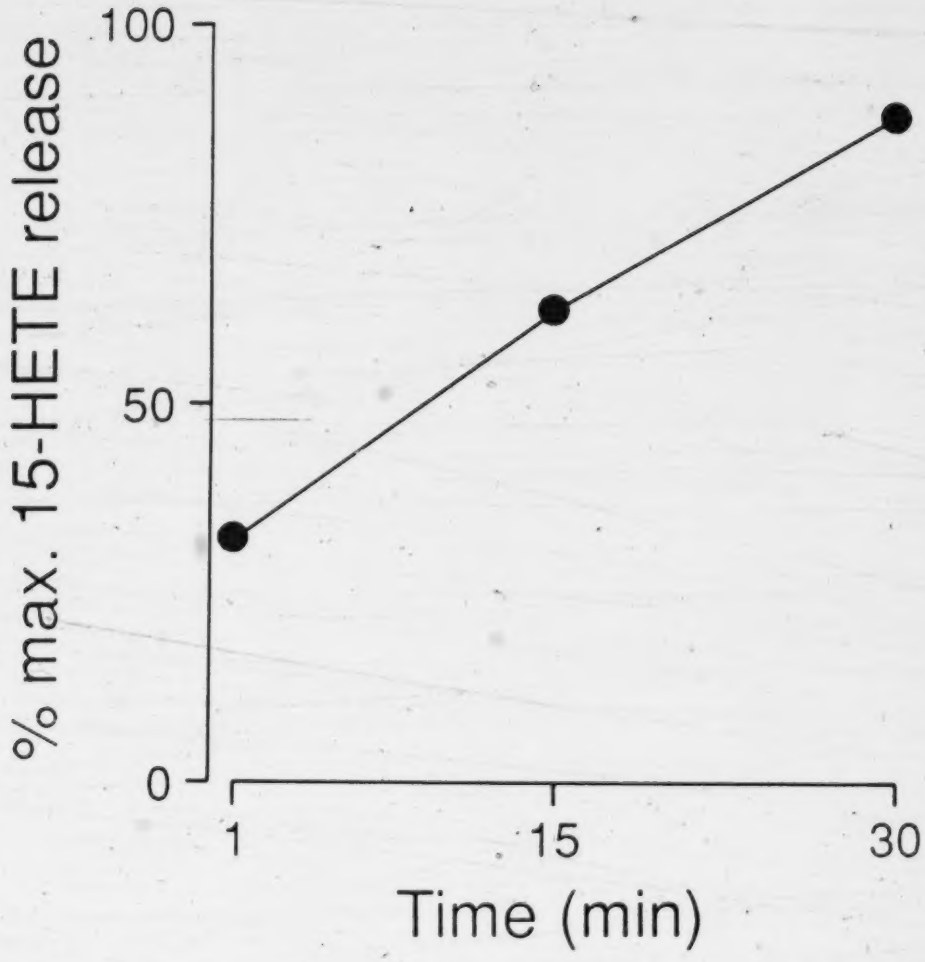


Fig. 3 Time course of 15-HETE release from cerebral artery smooth muscle cells, stimulated with arachidonic acid (10 μM). Each point expresses 15-HETE release as a percentage of the maximum value obtained, from duplicate determinations in two separate experiments. Mean absolute release of 15-HETE, corresponding to 100% release is 22.9 ± 6.5 pg/μg cell protein (N=4).

TABLE 1

Influence of drugs inhibiting arachidonic acid metabolism on 15-HETE release from cerebral artery smooth muscle cells[+]

	CONTROL		INDOMETHACIN (10μM)*			NDGA (10μM)**		
Expt.#	pg 15-HETE	Mean	pg 15-HETE	Mean	% control	pg 15-HETE	Mean	% control
1	75		72					
	94	85	72	72	84.7	-	-	-
2	110		110			68		
	123	117	75	93	79.5	72	70	59.8
3	112		104			66		
	80	96	75	90	93.8	56	61	63.5
4	204		176			188		
	234	219	202	189	86.3	155	172	78.5
5	146		151			165		
	160	153	167	159	103.9	90	128	83.7
			average		89.6			71.4
			± S.E.		± 4.2%			± 5.8%

[+] Duplicate wells of cells cultured in 12-well plates were incubated for 30 min in the presence or absence of drug and then with arachidonic acid (10 μM) for 15 min

* using absolute values as compared with control, not significant $p > 0.10$ (N=5)

** significant difference vs. control $p < 0.02$ (N=4)

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CHAPTER 8

CONCLUSIONS

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CONCLUSIONS

This thesis has described the metabolism of arachidonic acid in the cerebral artery wall, to products of both the cyclooxygenase and lipoxygenase pathways. The raison d'être of this work was to provide insight to the mechanism behind the pathogenesis of cerebral vasospasm, the delayed arterial narrowing seen a few days after the rupture of an intracranial aneurysm, which results in ischemic deficits due to the insufficient supply of blood to a portion of the brain. The major focus was to determine whether the lipoxygenase metabolites of arachidonic acid, including the leukotrienes and hydroperoxy- and hydroxy-eicosatetraenoic acids, could be formed by and act upon the cerebral circulation to influence vascular reactivity. The problem was viewed from three separate experimental perspectives: 1. using the primate model of cerebral vasospasm, to determine what the effect of direct exposure to subarachnoid clot for seven days in vivo would have on eicosanoid synthesis in the cerebral artery wall, 2. with isolated cerebral arteries, examining their in vitro pharmacological and biochemical activity in regards to the arachidonic acid cascade, and, 3. using a cell culture system which we developed for both smooth muscle and endothelial cells from cerebral arteries, applying this system to the investigation of lipoxygenase activity in a single cell type.

The major findings of this study can be summarized as follows:

1. The exposure of cerebral arteries to subarachnoid hemorrhage in a well established primate model of cerebral vasospasm does show that there is a disruption in cyclooxygenase metabolite formation in the

cerebral blood vessel wall. The predominant metabolite of this pathway, prostacyclin, synthesized primarily by the endothelium, is released in a diminished quantity from arteries which had been exposed to subarachnoid clot for seven days as compared to the situation where the clot was removed 24 hours after its placement. Early removal of blood clot from the subarachnoid space has a beneficial effect on cerebrovascular prostacyclin production. The consequences of early removal of blood clot on prostanoid release have not been tested in other animal models of vasospasm to date. Results using the canine model (Sasaki et al., 1981; Maeda et al., 1981; Walker et al., 1983) as well as those obtained with human arteries from patients with vasospasm (Walker et al., 1983) provide evidence for a decreased abilty of arteries in spasm to make this vasodilator component of blood vessel tone. In contrast no significant differences were observed between the clot, clot-removal and sham operated groups in terms of the ability of the excised cerebral arteries to synthesize thromboxane A_2, an eicosanoid with potent vasoconstrictor activity. Leukotriene C_4 did not appear to be released during the incubations, which were performed in a manner to simulate basal release conditions.

It is uncertain whether the diminished capacity of cerebral artery which was exposed to blood clot for seven days in vivo compared with arteries exposed for only 24 hours to release prostacyclin is simply a reflection of endothelial damage, which has been reported in arteries which had been in vasospasm (Liszczak et al., 1983), or an actively inhibited enzyme system in the intact cell. The relative contribution of prostacyclin in retaining the patency of the major cerebral arteries,

especially in the condition of vasospasm, is likely to be minor. The effectiveness of therapy of experimental vasospasm by the intravenous infusion of prostacyclin has been equivocal (Fukumori et al., 1983; White and Robertson, 1983). No resoundingly effective therapy of vasospasm has been put into practice with the use of agents which interfere with thromboxane synthesis or action (see Introduction). It appears that the prostacyclin-thromboxane imbalance hypothesis as proposed by Boullin et al. (1979) does not provide a complete explanation for the development of vasospasm.

2. It is now possible to culture smooth muscle cells from the major arteries of the cerebral circulation, using a non-enzymatic technique for the isolation and subculture of these cells. This may be advantageous in preserving in vitro characteristics of smooth muscle cells found in vivo. The cells express the most characteristic marker of vascular smooth muscle, smooth muscle α-actin (Vandekerckhove and Weber, 1978), even after multiple passage. In addition, the passaged cells possess functional voltage-dependent calcium and potassium channels characteristic of vascular smooth muscle, now making the study of ionic channels in cerebral artery smooth muscle more accessible, as this has only been done to date using freshly isolated cells. The study of calcium channels in cerebrovascular smooth muscle should be particularly interesting, as cerebral arteries are more susceptible than peripheral arteries to the effects of the calcium channel antagonists (Shimuzu et al., 1980) and these agents have shown some promise in decreasing the incidence of delayed ischemic deficits attributable to vasospasm (Allen et al., 1983).

The application of cell culture for the study of diseases such as atherosclerosis has allowed a better understanding of vascular wall biology. With this approach it is likely that some of the mysteries of the cerebral circulation may be unravelled. Cultured aortic smooth muscle cells were recently shown to undergo a progressive contraction and develop signs of necrotic damage over a seven day exposure to oxyhemoglobin (Fujii and Fujitsu, 1988). The effect of blood-derived products on cultured smooth muscle cells from cerebral arteries is relevant to the study of cerebral vasospasm, and can now be performed. Some initial experiments suggested that oxyhemoglobin can cause the release of 15-HETE (unpublished observations), more work is required. It is clear, however, that these cells possess lipoxygenase activity (vide infra).

3. It is possible to obtain pure cultures of endothelial cells from cerebral arteries, as was carried out in work reported here, without the use of enzymes, but improvements in the methodology are required in order to provide greater reproducibility. Necessary for this technique is a selective removal of the endothelium, to maximize the endothelial/smooth muscle cell ratio in the primary culture, as smooth muscle cells in culture inhibit the growth of endothelial cells (Orlidge and D'Amore, 1987). The use of collagenase does not ensure selective removal of the endothelium. Coating the culture dishes used for primary culture with a cell-selective substrate such as purified fibrinogen which enhances the attachment of endothelial but not smooth muscle cells may be one possibility in solving this difficulty (Dejana et al., 1987).

Much attention has been focused recently on the role the endothelium may play in the development of cerebral vasospasm, as these cells release both prostacyclin and endothelium-derived relaxation factor, two important dilators involved in the local regulation of blood vessel tone. Most recently a polypeptide named endothelin, having marked vasoconstrictor activity, has been isolated from peripheral artery endothelial cells (O'Brien et al., 1987; Yanagisawa et al., 1988). The effects of and the release of this substance in the cerebral circulation are unknown; findings such as this exemplify the remarkable regulatory role the endothelium has on blood vessel tone (Gordon, 1988). Improvements in the culture technique for endothelial cells from cerebral artery will allow these exciting new discoveries to be examined for their relevance to the cerebral circulation.

4. The sustained contraction of isolated rings of cerebral artery to arachidonic acid is not dependent upon the endothelium and is due to the formation of lipoxygenase metabolites of arachidonic acid. This is in contrast to the situation in peripheral arteries where generally an endothelium dependent contraction is seen which is blocked by inhibitors of cyclooxygenase (Miller and Vanhoutte, 1985; Singer and Peach, 1983). The finding that the response could only be partially inhibited by the leukotriene receptor antagonist FPL 55712 was an indication that other lipoxygenase products such as the HPETEs could be responsible for the contraction.

The findings by Shirahase et al. (1987) that thromboxane production is involved in the cerebral artery response to arachidonic acid is a different phenomemon, as they suggested that thromboxane is

important in the maintenance of resting tone. The authors used a spiral strip preparation of canine cerebral artery and noted a transient contraction to arachidonic acid; the results in Chapter 4 were performed on ring preparations from the same arteries and a sustained response was observed upon addition of arachidonic acid. Toda et al., (1988) using the same spiral strip preparation observed a transient contraction and relaxation after the addition of arachidonic acid. He suggested that the phasic contractile response was not due to the formation of thromboxane. He based this on the observation that the contraction effected by PGH_2, the prostaglandin endoperoxide which is the immediate precursor of thromboxane, was not influenced by OKY-046, a thromboxane synthetase inhibitor. These studies suggest that the short-lived contraction observed to arachidonic acid is endothelium-dependent and via the formation of cyclooxygenase metabolites; we observed a tonic response which is clearly a distinct observation as it was not affected by the presence of inhibitors of cyclooxygenase.

5. Cerebral arteries have the capability to synthesize LTC_4 and LTD_4 when stimulated with the calcium ionophore A23187. Lindgren et al. (1984) initially observed the release of peptidoleukotrienes from rat brain slices and suggested that neuronal tissue was the primary source of such products whereas Kiwak et al. (1985) showed that gerbil cerebral arteries can release more immunoreactive SRS-A activity per unit weight than neuronal tissue. Piper et al. (1988) have recently reported that the cerebral arteries from the pig have much greater LTD_4-like synthetic activity than arteries taken from the peripheral

circulation. The involvement of these vasoconstrictor leukotrienes in cerebral vasospasm is speculative. An enhanced cerebrospinal fluid level of LTC_4 has been observed in patients with subarachnoid hemorrhage showing signs of vasospasm as compared to those without arterial narrowing (Paoletti et al., 1988).

6. When isolated segments of cerebral artery are incubated with exogenous arachidonic acid, the major lipoxygenase products appear to be 15- and 5-HPETE, measured as their reduced congeners the HETEs; no leukotrienes were observed by HPLC. This need not conflict with the results observed in the contractility study (Chapter 4) where some peptidoleukotriene was released upon incubation with arachidonic acid, as a more sensitive radioimmunoassay was used for analysis, which had a tenfold or more greater sensitivity than HPLC. However it is possible that the HPETEs may account for the major portion of the contractile response of isolated canine cerebral arteries to arachidonic acid, in terms of the quantity in which can be released, their potency, and their efficacy, all of which are greater than the leukotrienes (Koide et al., 1982; Asano et al., 1983; Tagari et al., 1983).

Chapter 7 in this thesis shows that cerebral artery smooth muscle cells release primarily 15-HETE upon incubation with arachidonic acid, giving additional evidence that 15-HPETE may be responsible for the endothelium-independent contraction of cerebral arteries to arachidonate. Studies with lipoxygenase inhibitors do not conflict with the data, as these drugs (such as NDGA) are generally not specific with respect to blockade of 15- versus 5-lipoxygenation. In the study describing AA-861 as a selective 5-lipoxygenase inhibitor (Yoshimoto et

al., 1982), the authors did not test the effect of the drug on 15-lipoxygenase. Formation of both 5- and 15-HETE in cerebral artery was markedly reduced in the presence of AA-861. This agent was effective in reducing the severity of vasospasm seen one week after two injections of blood in the subarachnoid space of dogs (Yokota et al., 1987), thus its ameliorative action could be attributed to the reduction of metabolites generated from both the 5- and 15-lipoxygenase pathways.

These studies of the activity of the lipoxygenase enzymes in normal cerebral arteries and smooth muscle cells cultured from the same source have brought to light the ability of the vessel wall to produce these eicosanoids which have impressive activity in the cerebral vasculature. What is less certain is whether they play a role in the development of vasospasm. It is worth remembering that endogenous autacoids such as serotonin, which has a pronounced contractile effect on the cerebral vasculature and is possibly released during blood clot formation after the rupture of the aneurysm, is generally believed to have little to do with delayed vasospasm. On the other hand, these lipoxygenase metabolites possess all the criteria required for agents involved in vasospasm: i) they are present in some part of the vascular circulation (the blood vessel wall, neuronal tissue, cells of the blood clot), ii) they are normally found in an inactive form (as arachidonic acid which is the most prevalent of the fatty acids making up the phospholipid bilayer of the cell membrane, and iii) there is a plausible mechanism for their release following subarachnoid hemorrage (by tissues traumatized from the aneurysmal rupture; from

blood cells as part of the clotting mechanism or during the process of clot removal; from the release of free radical species during the degradation of oxyhemoglobin released from the clot or from cells recruited to clean up the clot, setting up a chain reaction of lipid peroxidation which in turn activates lipoxygenase (Rouzer and Samuelsson, 1986)).

The most attractive facets of a lipoxygenase theory of cerebral vasospasm is that the HPETE/HETEs and leukotrienes possess more than just contractile activity on the blood vessels, as an active pharmacological contraction could not persist over such a time course without some form of tachyphylaxis occurring, and that features of their release approximate the time course of the development of the delayed vasospasm. A slow formation of HPETE during the process of blood clot degradation in the first days following the subarachnoid hemorrhage could be involved in changes such as inhibition of normal prostacyclin release by the endothelium and a cause of necrotic damage in the vascular wall as well as activating or potentiating leukotriene formation. The size and extent of the blood clot would be in proportion to the extent of lipoxygenase product release, and depending upon how rapidly the body's mechanisms provide for removal of the clot, the vascular wall underlying the clot may be spared or damaged at such a rate that vascular repair cannot keep up with it, resulting in the critical delayed narrowing, which is unresponsive to pharmacological treatment.

Research in the field of cerebral vasospasm over the last thirty years has yielded a lot of evidence of what is not involved in the

pathogenesis of the disorder. Further studies, especially using a suitable animal model, will help explore the validity of this hypothesis. The use of cell culture systems as a new tool in this area will allow for a clearer understanding of the plethora of events which in combination must result in the full clinical picture of this mysterious and devastating disorder.

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